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Award Number: W81XWH-08-1-0143

TITLE: Obstructing Androgen Receptor Activation in Prostate Cancer Cells Through Post-translational Modification by NEDD8

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REPORT DATE: Þ[ç^{ à^¦Á Œ–FG

TYPE OF REPORT: Œ} }a

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
Þ[ç^{ à^¦ÁGEFG	Øã}æ|	15 April 200Ì – 14 U&¦à^¦ 201G

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Obstructing Androgen Receptor Activation in Prostate Cancer Cells Through Post-translational Modification by NEDD8	
	5b. GRANT NUMBER W81XWH-08-1-0143
	5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)	5d. PROJECT NUMBER
J. Don Chen, Ph.D.	
	5e. TASK NUMBER
E-Mail: chenjd@umdnj.edu	**5f. WORK UNIT NUMBER**

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
Robert Wood Johnson Medical School Piscataway, NJ 08854	

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

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15. SUBJECT TERMS

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16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	Îï	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

INTRODUCTION

Prostate cancer is second to lung cancer in annual cancer death and strikes as many men as breast cancer does in women. Androgen receptor (AR) is a member of the steroid/nuclear receptor (NR) superfamily and plays a pivotal role in the progression of prostate cancer. As androgen promotes the growth of prostate tumors and AR is expressed throughout various caner stages, it is of particular importance to explore regulatory mechanisms that control AR activity.

Transcriptional activation by NRs involves sequential and combinatorial interactions with multiple cofactors, some of which are directly involved in chromatin modification and remodeling. According to the histone code model, reversible post-translational modifications such as methylation, acetylation, phosphorylation, and ubiquitination achieved by opposing enzyme pairs correlate with the specific activated or repressed status of involved promoters. In addition to histone tails, transcriptional factors *per se* are also subjected to those modifications. Deposition and removal of post-translational marks greatly diversify the transcription factor with a strong portfolio to handle different cellular stimuli. NEDD8 is an ubiquitin-like modifier that was reported to modify p53 among a few other proteins. Neddylation of p53 inhibits its transcription activity; however, the molecular mechanism remains unclear.

The protein inhibitor of activated STAT-y (PIASy) was recently reported to exhibit inhibitory effects on AR's transcriptional activity. PIASy is a member of the PIAS family and represses several transcriptional factors, including LEF1, c-Myb, GATA-2, and p53. The mechanism of PIASy's transcriptional inhibitory effect is presumably mediated through a sumoylation-dependent pathway. However, the mechanism by which PIASy inhibits AR's transactivation remains unknown.

The Jun activation domain binding protein-1 (Jab1, also known as CSN5 or COPS5) is connected to various biological responses as it interacts with multiple signaling molecules such as c-Jun, p27, MIF, HIF1a, Smad4, p53 and cullin1. Jab1 is the fifth component of the COP9 signalosome (CSN) complex and contains a conserved Jab1/MPN domain metalloenzyme (JAMM) motif. The JAMM motif plays a critical role in CSN-mediated deneddylation pathway and it further regulates the activity of Cullin/Rings domain dependent E3 Ligase (CRL)-like complex. Nullification of Jab1 resulted in embryonic death soon after implantation and the Jab$^{-/-}$ cells significantly accumulated p27, p53, and cyclin E, causing impaired proliferation and accelerated apoptosis. Recently, Jab1 was linked to the estrogen pathway through enhancing estradiol-dependent degradation of ERa in the cytoplasm. Since amplification of the Jab1 locus region near chromosome 8q was frequently found in prostate cancer patients, it is imminent to determine whether and how Jab1 might affect AR activity.

In this study, we have uncovered a new reversible mechanism achieved by PIASy and Jab1 that regulates AR's transcriptional activity: namely neddylation. As a consequence, down-regulation of Jab1 hinders androgen-dependent gene expression and prostatic tumor cell growth, thereby offering a potential therapeutic target for

treating prostate cancer. These findings are summarized in the BODY section shown below. Additional details including references and figures for this study are shown in a manuscript in Appendix 1.

In addition, we have also completed an extra task that was not described in the original plan and that was in part supported by this grant. This work involves the alternation/deficiency in activation-3 (ADA3) protein, which is an essential component of the human PCAF and yeast SAGA/ADA histone acetyltransferase complexes. These complexes facilitate transactivation of target genes by association with transcription factors and modification of local chromatin structure. It is known that the yeast ADA3 is required for nuclear receptor (NR)-mediated transactivation in yeast cells; however, the role of mammalian ADA3 in NR signaling remains elusive. We have investigated how the human ADA3 regulates retinoic acid receptor (RAR) α-mediated transactivation. We show that hADA3 interacts directly with RARα in a hormone-dependent manner, and this interaction contributes to RARα transactivation. Intriguingly, this interaction involves classical LxxLL motifs in hADA3, as demonstrated by both "loss" and "gain" of function mutations, as well as a functional coactivator pocket of the receptor. Additionally, we show that hADA3 associates with RARα target gene promoter in a hormone-dependent manner. Furthermore, a structural model was established to illustrate an interaction network within the ADA3/RARα complex. Our results suggest that hADA3 is a bona fide transcriptional coactivator for RARα, acting through a conserved mechanism involving direct contacts between NR boxes and the receptor's coactivator pocket. Details of this study can be found in a reprint shown in Appendix 2.

BODY

Task 1-1. Month 1-3: We planned to develop protocols to enrich neddylated form of endogenous AR. We wished to demonstrate evidence that a portion of endogenous AR is modified by NEDD8.

We have successfully developed a protocol to enrich neddylated form of endogenous AR. We have demonstrated evidence that a portion of the endogenous AR is indeed modified by Nedd8. We tested various protocols to enrich neddylated form of endogenous AR. First, large quantity of endogenous AR from LNCaP cells was purified by double immunoprecipitation with anti-AR and anti-NEDD8 antibodies. The neddylated form of AR was detected on Western blot using anti-AR and anti-NEDD8 antibodies (Figure 1). We have also tested various growth conditions to determine their effects on neddylation of endogenous AR in LNCaP cells. Preliminary data suggest that AR can be neddylated under several conditions.

Figure 1. Endogenous AR is modified by NEDD8.
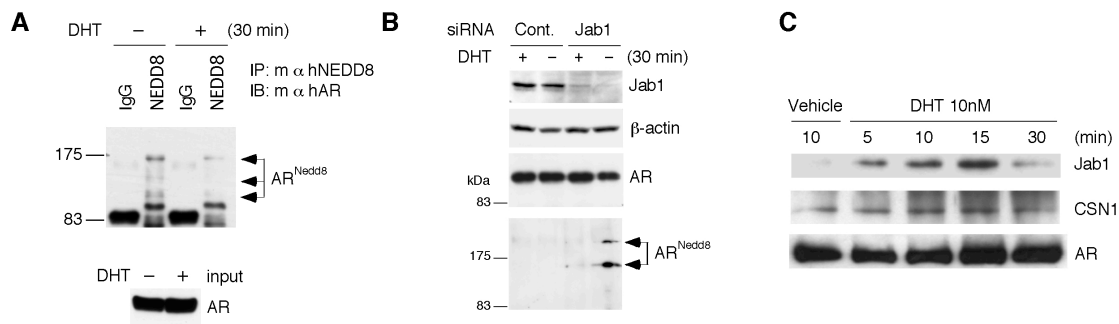
LNCaP cells were maintained in stripped media for at least four days and endogenous neddylated AR was detected by immunoprecipitating neddylated proteins with mouse anti-NEDD8 antibody and immunoblotted by mouse anti-AR antibody (Figure 1A). We found that neddylation of endogenous AR is enhanced in Jab1 knockdown cells (Figure 1B). In LNCaP cells with Jab1 silencing, immunoprecipitation was conducted by mouse anti-AR antibody and detected by rabbit anti-NEDD8 antibody. Knockdown of endogenous Jab1 was verified by Western blotting. b-actin serves as a loading control. Furthermore, we found evidence of association of Jab1 and CSN1 with AR upon DHT treatment (Figure 1C). CWR22Rv1 prostate cancer cells stably transfected with S-tag hAR were treated with solvent (ethanol) or DHT (10 nM) for indicated time period. AR complexes were purified using S protein column, resolved on SDS-PAGE and detected by goat anti-Jab1 or rabbit anti-CSN1 antibodies, respectively.

Task 1-2. Month 3-6: We planned to compare AR neddylation in various prostate cancer cell lines including LNCaP and PC3. We wished to provide data showing how AR neddylation may vary among different type of cell lines.

Preliminary data suggest that AR neddylation may occur in different cell types. We have not observed significant differences among different prostate cancer cell types.

Task 1-3. Month 6-9: We planned to determine the effect of androgen treatment on neddylation of endogenous AR. We wished to answer whether androgen treatment indeed blocks neddylation of AR, and we wished to provide discussion of how this might occur.

We have completed this task. As shown in Figure 1A and B, AR neddylation shows clear reduction upon treatment with DHT.

Task 2-1. Month 9-12: We planned identify potential neddylation consensus sequences within AR. We wished to identify potential amino acid sequences in AR that may serve as conjugation sites for NEDD8.

We have identified K630 as a potential neddylation site on AR. The results are shown in Figure 2.

Figure 2. PIASy–mediated neddylation of wild type AR (lane 5) was greatly diminished in both K630R and 3KR mutants (lanes 6 and 7). His-ISG15 was used as a negative control (lane 8). The Western blots of ectopic expression levels of AR and PIASy were determined, as well as b-actin as a loading control (lower panel).
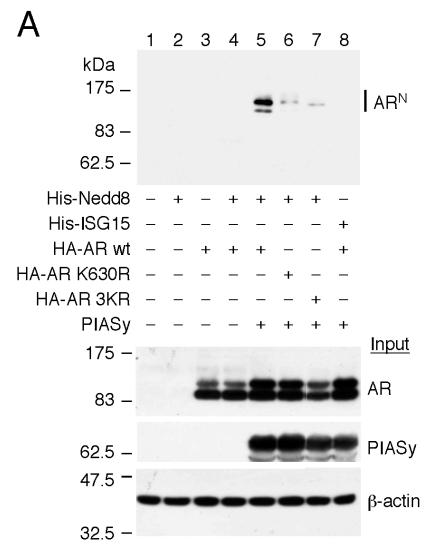
Task 2-1. Month 9-12: We planned to identify potential neddylation consensus sequences within AR. We wished to identify potential amino acid sequences in AR that may serve as conjugation sites for NEDD8.

We have identified K630 as a potential neddylation site on AR as described in the year 1 annual report.

Task 2-2. Month 12-15: We planned to test individual lysine residues to determine the precise neddylation sites. Ultimately, we will provide evidence that specific amino acids are sites of NEDD8 modification.

We have tested individual lysine residues and determined the neddylation sites. By comparing reported NEDD8 substrates such as Cullins and p53, a similar stretch of lysine residues was found at the hinge region of AR (K630, K632 and K633). To determine whether these lysine residues are involved in AR neddylation, we created K630R and K630R/K632R/K633R (3KR) mutants and analyzed neddylation status of these mutants. Ni^{2+}-NTA agarose beads pull down assay revealed that the amount of neddylated AR was greatly diminished in both mutants. As the 3KR mutant retained residual neddylation comparable to that of the K630R mutant, the data suggest that the K630 residue may be most critical for AR neddylation.

Task 3-1. Month 15-18: We planned to create AR mutants that are deficient in NEDD8 modification. These AR mutants will be available for future studies.

We have created AR mutants that are deficient in modification by NEDD8. Since the K630R and 3KR mutants of AR were defective in neddylation, they offer an opportunity to determine the contribution of neddylation to PIASy-mediated transcriptional inhibition. Provided that PIASy inhibits AR activity through K630 neddylation, then both K630R and 3KR mutants should be resistant to PIASy-mediated inhibition. In a transient transfection assay, we found that both K630R and 3KR mutants were capable of activating reporter gene expression comparable to the wild type AR, yet PIASy could only inhibit the wild-type AR, but not the K630R or 3KR mutant. These results support the conclusion that neddylation of AR at distinct lysine residues may be the mechanism whereby PIASy inhibits AR's transcriptional activity.

Task 3-2. Month 18-21: We planned to test various AR neddylation mutants for other types of modification. We wished to determine how neddylation is involved in other types of post-translational modifications.

We have tested our AR neddylation mutants for other types of post-translational modifications. So far have not found evidence of advert effect on other types of modifications.

Task 3-3. Month 21-24: We planned to determine the effect of AR neddylation mutants on transcriptional activation and promotion of prostate cancer cell growth and proliferation. We wished to provide evidence to determine whether AR neddylation affects transcriptional activation of AR and/or prostate cancer cell growth and proliferation.

We found that AR neddylation mutants are defective in transcriptional activation and in promotion of prostate cancer cell growth and proliferation.

In addition to these proposed tasks, we have also conducted *In vitro* neddylation assay by mixing ^{35}S-labeled AR with purified APP-BP1/Uba3 (E1), Ubc12 (E2), NEDD8, ATP and His-PIASy. The autoradiography shows that PIASy catalyzes AR neddylation in a time course-dependent manner. In vitro neddylation assay also demonstrated that PIASy stimulates AR neddylation in a concentration-dependent manner. Furthermore, we also demonstrated that PIASy-mediated neddylation and transcriptional inhibition are conserved in both ERa and PR.

Task 4-1. We proposed to generate prostatic cancer cell lines with expression of shRNA against Jab1 or scramble control shRNA. Plasmids encoding the shRNA sequences against endogenous Jab1 were constructed into the pLentiLox 3.7 vector under the control of an U6 promoter. The Jab1 shRNA LentiLox 3.7 vector was mixed with the ViralPower^{TM} Packaging Mix (containing pLP1, PLP2 and pLP/VSVG DNAs) and used for transfection into 293T cells. After viral particles were packaged and released, culture media were harvested to infect LNCaP and PC3 cell lines and silencing effect by shRNA were determined by detecting the diminishing protein levels of endogenous Jab1. Furthermore, the mRNA and protein levels of PSA or Probasin, which are known AR target genes, were characterized after Jab1 silencing.

We are pleased to report that several Jab1 shRNA expressing lines have been successfully established.

Task 4-2. We proposed to characterize the effects of endogenous Jab1 silencing on protein stability of p53 and p27 and cell growth. The effects of Jab1 silencing on the cell proliferation were determined by MTT assay. We plated both LNCaP and PC3 with Jab1 silencing or control at $1X10^4$ cells per well in a 96 well microtiter plate, and the effects on cell proliferation and growth curve were analyzed. We expect that silencing endogenous Jab1 will impede on cell proliferation of both LNCaP and PC3 cells. To understand growth inhibition mediated by Jab1 silencing, cell cycle analyses will also be

conducted to determine which phase of cell cycle was arrested. Sample will be collected and subjected to FACS flow cytometer analysis. In addition, we will measure the steady state protein level of p53, p21, p27, and pRb. In the Jab1 silencing cell lines, we expect protein level of p53, p21 and p27 will be accumulated and pRB will be hypo-phosphorylated.

We are happy to report that silencing of endogenous Jab1 affects protein stability of p53 and p27 and cell growth. The effects of Jab1 silencing on the cell proliferation were determined by MTT assay. We found that silencing Jab1 impede on cell proliferation of both LNCaP and PC3 cells. To understand growth inhibition mediated by Jab1 silencing, cell cycle analyses were conducted to determine which phase of cell cycle was arrested. So far, the evidence is inconclusive, and we found little evidence of Jab1 silencing on affecting protein stabilities of p53 and p27.

KEY RESEARCH ACCOMPLISHMENTS:

Year 1
- We have developed a protocol to enrich neddylated form of endogenous AR.
- We have demonstrated evidence that a portion of endogenous AR is modified by NEDD8.
- We have compared AR neddylation in various prostate cancer cell lines including LNCaP and PC3.
- We have found no evidence that AR neddylation varies among different type of cell lines.
- We have determined the effect of androgen treatment on neddylation of endogenous AR.
- We found that androgen treatment may block neddylation of AR.
- We have identified potential neddylation site within AR.

Year 2
- We have tested individual lysine residues and determined the neddylation sites.
- We have created AR mutants that are deficient in being modified by NEDD8.
- We have tested our available AR neddylation mutants for other types of post-translational modifications.
- We are in the process of determining the effect of AR neddylation mutants on transcriptional activation and promotion of prostate cancer cell growth and proliferation.
- we have also conducted *In vitro* neddylation assay and shows that PIASy catalyzes AR neddylation in a time course and concentration-dependent manner.
- Furthermore, we also demonstrated that PIASy-mediated neddylation and transcriptional inhibition are conserved in both ERa and PR.

Year 3
- We have established several Jab1 shRNA expressing cell lines in LNCaP and PC3 cells.

- We have partially characterized the effect of Jab1 silencing on cell growth and proliferation of prostate cancer cells.

Year 4
- We have established several Jab1 shRNA expressing cell lines in LNCaP and PC3 cells.
- We have characterized the effect of Jab1 silencing on cell growth and proliferation of prostate cancer cells and found that silencing of Jab1 inhibits prostate cancer cell growth.

REPORTABLE OUTCOMES:

1. Chang, K. H., Hsiao, P.-W. and Chen, J. D. Modulation of Androgen Receptor Activity by Reversible NEDD8 Modification. (under revision)

2. Li, C.-W., Ai, N., Dinh, G. K., Welsh, W. J. and Chen, J. D. (2010) Human ADA3 Regulates RARα Transcriptional Activity through Direct Contact between LxxLL Motifs and the Receptor Coactivator Pocket. *Nucl. Acid Re*search. 38: 5291-303.

CONCLUSION:

In summary, we have completed virtually all proposed tasks in this study. We have further completed an additional related task. Several key accomplishments and reportable outcomes are listed above. We expect to continue the research in future studies.

REFERENCES:

(see Articles in Appendices)

APPENDICES:

1. MCB Manuscript.
2. Nucleic Acid Research paper.

SUPPORTING DATA:

(see manuscripts)

Appendix 1.

Chang, K. H., Hsiao, P.-W. and Chen, J. D. Modulation of Androgen Receptor Activity by Reversible NEDD8 Modification. (under revision)

Modulation of Androgen Receptor Activity by Reversible NEDD8 Modification

Kai-Hsiung Chang[1], Pei-Wen Hsiao[2], and J. Don Chen[1][*]

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Abbreviation: AR, androgen receptor; NEDD8, neural expressed development down-regulated 8; PIAS, protein inhibitor of activated STAT; Jab1, Jun activation domain binding protein 1; JAMM, Jab1/MPN domain metalloenzyme motif; CSN, COP9 signalosome; ER, estrogen receptor; CRL, Cullin/Rings domain dependent E3 Ligase; SCF, Skp_cullin_F-Box complex.

Running Title: Neddylation Regulates AR Activity

ABSTRACT

Androgen receptor (AR) is a crucial ligand-dependent transcription factor with the androgen-AR axis as a main target for treating prostate cancer. While post-translational modification on both histones and transcriptional factors has significant impacts on gene expression, how it affects AR's activity is largely unknown. Here we show that AR is covalently modified and functionally regulated by NEDD8, an ubiquitin-like small protein modifier. We show that PIASy acts as an E3 ligase catalyzing AR neddylation and inhibiting its transcriptional activity. Importantly, the PIASy-mediated neddylation and transcriptional inhibition also apply to other nuclear receptors including PR and ER. Conversely, Jab1 interacts with AR and enhances AR's transcriptional activity through its deneddylation activity. In agreement, knockdown of Jab1 increases the neddylation level of endogenous AR in prostate cancer cells and hinders androgen-induced gene activation, as well as impeding prostate cancer cell growth. Taken together, these results suggest that reversible neddylation is important for the regulation of AR activity, providing a new potential target for managing prostate cancer.

INTRODUCTION

Prostate cancer is second to lung cancer in annual cancer death and strikes as many men as breast cancer does in women. Androgen receptor (AR) is a member of the steroid/nuclear receptor (NR) superfamily and plays a pivotal role in the progression of prostate cancer (29). As androgen promotes the growth of prostate tumors and AR is expressed throughout various caner stages, it is of particular importance to explore regulatory mechanisms that control AR activity.

Transcriptional activation by NRs involves sequential and combinatorial interactions with multiple cofactors, some of which are directly involved in chromatin modification and remodeling (13). According to the histone code model (5, 16, 26), reversible post-translational modifications such as methylation, acetylation, phosphorylation, and ubiquitination achieved by opposing enzyme pairs correlate with the specific activated or repressed status of involved promoters. In addition to histone tails, transcriptional factors *per se* are also subjected to those modifications. Deposition and removal of post-translational marks greatly diversify the transcription factor with a strong portfolio to handle different cellular stimuli. NEDD8 is an ubiquitin-like modifier that was reported to modify p53 among a few other proteins (32). Neddylation of p53 inhibits its transcription activity; however, the molecular mechanism remains unclear (32).

The protein inhibitor of activated STAT-y (PIASy) was recently reported to exhibit inhibitory effects on AR's transcriptional activity (14). PIASy is a member of the PIAS family and represses several transcriptional factors, including LEF1, c-Myb, GATA-2, and p53 (6, 10, 21, 23). The mechanism of PIASy's transcriptional inhibitory effect is presumably mediated through a sumoylation-dependent pathway (6, 10, 21, 23). However, the mechanism by which PIASy inhibits AR transactivation remains unknown.

The Jun activation domain binding protein-1 (Jab1, also known as CSN5 or COPS5) is connected to various biological responses as it interacts with multiple signaling molecules such as c-Jun, p27, MIF, HIF1α, Smad4, p53 and cullin1 (1, 2, 7, 15, 19, 27, 30). Jab1 is the fifth component of the COP9 signalosome (CSN) complex and contains a conserved Jab1/MPN domain metalloenzyme (JAMM) motif. The JAMM motif plays a critical role in CSN-mediated deneddylation pathway and it further regulates the activity of Cullin/Rings domain dependent E3 Ligase (CRL)-like complex (8, 9). Nullification of Jab1 resulted in embryonic death soon after implantation and the Jab^{-/-} cells significantly accumulated p27, p53, and cyclin E, causing impaired proliferation and accelerated apoptosis (28). Recently, Jab1 was linked to the estrogen pathway through enhancing estradiol-dependent degradation of ERα in the cytoplasm (4). Since amplification of the Jab1 locus region near chromosome 8q was

4

frequently found in prostate cancer patients (25), it is imminent to determine whether and how Jab1 might affect AR activity.

Herein we present a new reversible mechanism achieved by PIASy and Jab1 that regulates AR's transcriptional activity: namely neddylation. As a consequence, down-regulation of Jab1 hinders androgen-dependent gene expression and prostatic tumor cell growth, thereby offering a potential therapeutic target for treating prostate cancer.

MATERIALS AND METHODS

Plasmids and Antibodies

The luciferase reporters PSA-LUC, PS2-LUC and MMTV-LUC and expression plasmids of wild-type AR, PR-B, ERα and β-galactosidase were previously described (17, 33). The p53, Mdm2, and 6XHis tag fused NEDD8 and ISG15 were kindly provided by Dr. Ronald Hay (32). The full-length Jab1 and PIASy were purchased as an I.M.A.G.E. clone from ATCC and cloned into pCMX, pET (Novagen), and pGEX vectors (Amersham Biosciences). The Jab1 fusion proteins GST-Jab1 (1-190), GST-Jab1 (191-334), and GST-Jab1 (1-272) were created by PCR with sequence-specific primers and sub-cloned into the pGEX vector. The GST-Jab1 dMPN, Jab1 D151N, AR-K630R, AR-3KR, PIASy-C342A, and PIASy-C347A were generated by using QuickChange® II XL site-direct mutagenesis kit followed the manufacturer's instructions (Stratagene, La Jolla,

5

CA). The integrity of all constructs was confirmed by sequence analyses.

The rabbit anti-HA polyclonal antibody (1:2000) and mouse anti-Flag (M2) monoclonal antibody (1:4000) was purchased from MBL and Stratagene, respectively. The rabbit anti-NEDD8 polyclonal antibody (1:1000) was from Chemicon. The mouse anti-β-actin monoclonal antibody (1:5000), mouse anti-AR monoclonal antibody (1:2500), rabbit anti-CSN1 polyclonal antibody (1:1000), goat anti-Jab1 polyclonal antibody (1:1200), and goat anti-PIASy polyclonal antibody (1:1000) were from Santa Cruz Biotechnology. The rabbit anti-AR (PG-21) polyclonal antibody (1:2000) was from Upstate, Inc.

Cell Culture, Transfection, Luciferase and GST Pull-down Assays
The prostate cancer cell line LNCaP and CWR22Rv-1 were cultured in RPMI-1640, and HEK293 and Monkey COS7 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) (GibcoBRL, Carlsbad, CA). For experiments treated with steroid hormones such as DHT, estradiol, or progesterone, cells were incubated with phenol red-free medium containing 10% charcoal-dextran stripped FBS (stripped growth medium) at least two days prior to experimentation. Luciferase reporter assays were conducted as described (33). Purified GST-fusion proteins of full-length Jab1 as well as mutants with different fragments were prepared as previously described (12).

6

Lentivirus-Mediated RNA Interference

RNA silencing of endogenous Jab1 has been described previously (3). DNA encoding the shRNA sequences against Jab1 or a scramble control were cloned into the LentiLox 3.7 vector (a kind gift obtained from Dr. Van Parijs' lab at MIT). The lentiLox 3.7 is designed to express shRNA under the control of the U6 promoter and to allow rapid selection of stably infected mammalian cells with CMV promoter-driven expression of EGFP as a selection marker. The Jab1 or control shRNA LentiLox 3.7 vector (pLL 3.7-Jab1 or pLL3.7-control) were mixed with the ViralPower™ Packaging Mix (containing pLP1, PLP2 and pLP/VSVG DNAs, which provide the replication and structure proteins) and transfected into 293T cells by calcium phosphate precipitation for 12 hours. After replacing fresh media for 24 hours, media containing released viral particles were harvested to infect HEK293 and LNCaP cell lines. The silencing effect by shRNA was verified by detecting the protein level of endogenous Jab1 using Western blot. To generate colonies, diluted lentivirus-infected cells (around 10^{2}) were split into 10 cm dish and allowed to grow for 1-2 weeks. To pick colonies, prepare 24-well plates with 1 ml media in each well. Colonies with the expression of EGFP are marked under an invert fluorescence microscope. The colonies of interest were isolated using a Gilson pipette with a sterile yellow tip and then transfer it to a well in a 24-well plate. Subsequent split and expansion were performed and the silencing effect of Jab1 was determined by Western blot.

Co-immunoprecipitation

COS7 cells (3X10^7) transfected with pCMXHA-hAR and pCMXFLAG-Jab1 for 24 hours were lysed in NP-40 lysis buffer (20 mM HEPES pH 7.9, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 10 mM glycerol phosphate, 10 mM sodium pyrophosphate, 1 mM dithiothreitol, protease inhibitor cocktail (Roche), 1 mM NaF, 1 mM Na$_3$VO$_4$, 0.1% Nonidet P-40). Pre-cleared with 25 µl protein-A/G agarose beads, 700 µg lysate was subjected to immunoprecipitation with 20 µl anti-FLAG antibody conjugated agarose beads (M2 beads) or anti-HA antibody conjugated agarose beads (Sigma) at 4 °C for 3 hours. The beads were collected by centrifugation and washed five times with lysis buffer. The immunoprecipitated complex were boiled in 25 µl 2X SDS sample buffer and analyzed by 5-15% SDS-PAGE. The transferred PVDF membrane was incubated with appropriate first and second antibodies and followed by detection with chemiluminescence (ECL, Amersham Biosciences).

For S-protein pull down assays, S-tag fused full-length hAR was transfected into CWR22Rv-1 and stable clone was selected with media containing G418. Cells (3X10^7) were transiently treated with 10 nM DHT or ethanol as vehicle control for 10 minutes or indicated time periods and then harvested and lysed in NP-40 lysis buffer. About 1500 µg pre-cleared lysate were incubated with S-protein at 4 °C for 3 hours. The beads containing AR complex were collected by centrifugation and washed five times with lysis buffer. The boiled precipitated complex was revolved

by 5-15% SDS-PAGE and gels were silver stained according to manufacturer's recommendations (Pierce) or transferred onto PVDF membrane and subject to Western blotting.

Purification of Proteins Modified by NEDD8

LNCaP cells infected with lentivirus-mediated RNA interference against Jab1 or control lentivirus were cultured with stripped growth media for 48 hours. After treatment with 10 nM DHT or ethanol for 30 minutes, endogenous protein conjugated with NEDD8 was purified as describe before (32). For purification of 6XHis-NEDD8 conjugated proteins, experiments were conducted as previously described with minor modifications (22, 31). Briefly, Monkey COS7 or HEK293 cells were cotransfected with pcDNA3-6XHis-NEDD8, pCMXHA-hAR and pCMV-PIASy. Post-transfection cells (36 hours) were harvested. Twenty percent of cell suspension in PBS was lysed by NP-40 lysis buffer and ectopic expressed proteins were analyzed by Western blot with appropriate antibodies. The remainder was lysed with 4ml lysis buffer (6 M Guanidine-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris/HCl, pH 8.0, 5 mM imidazole, and 10 mM β-mercaptoethanol). Proteins covalently conjugated by 6XHis-NEDD8 are pulled down by 40 μl Ni-NTA-agarose (QIAGEN) for 2 hours at room temperature and successively washed by the following buffer: 6 M Guanidine-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris/HCl, pH 8.0, 5 mM imidazole plus 10 mM β-mercaptoethanol; 8 M Urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris/HCl, pH 8.0, 10 mM imidazole, 10 mM β-mercaptoethanol plus 0.1% Triton X-100; 8 M

Urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris/HCl, pH 6.3, 10 mM β-mercaptoethanol (buffer A), 20 mM imidazole plus 0.2% Triton X-100 twice; buffer A with 10 mM imidazole plus 0.1% Triton X-100; buffer A with 10 mM imidazole plus 0.05% Triton X-100. After last wash, the proteins were eluted by 2X SDS sample buffer containing 200 mM imidazole and then analyzed by SDS-PAGE and Western blotting.

Cell proliferation and Soft Agar Colony assay

The pLL3.7-Jab1 and pLL3.7 control were used to generate recombinant lentiviruses to infect LNCaP cells. Infected cells were recovered for 3 days in RPMI-1640 media with 10% FBS. Cell proliferation was measured by counting the cell numbers with trypan blue stain in a hemocytometer. Briefly, LNCaP cells infected with pLL3.7-Jab1 or pLL3.7-control were plated in 12-well plates at a density of 10^5 cells/well in 1ml RPMI-1640 with 10% FBS. The cells were incubated at 37 °C in a humidified 5% CO_2 incubator. Next day, the cells were trypsinized and stained with 0.08% trypan blue. Viable cell numbers were counted for 6 days and a growth curve was plotted. The experiments were repeated three times in triplet.

Assays of colony formation on soft agar were performed using standard protocol. Briefly, 2-ml base layers containing 0.6% agar medium were prepared in a 6-well plate by mixing equal volumes of 1.2% Noble agar with 2X RPMI-1640 medium with 20% FBS. LNCaP cells infected with lentivirus containing shRNA against Jab1 or

control were trypsinized, centrifuged and re-suspended in 0.3% agar medium containing 10% FBS. 10^4 cells/ml/well were plated onto the previously prepared base layers and incubated in a humidified 5% CO_2 incubator for 4 weeks. Colonies were photographed, counted, and analyzed by Quality One® software from *BIO-RAD*. The experiments were repeated three times in triplet.

RESULTS

PIASy Inhibits AR Transcriptional Activity Through Neddylation

PIASy has been reported to inhibit transcription through catalyzing SUMO-1 modification on several transcription factors (6, 10, 21, 23), while it also inhibits AR transcriptional activity, but through an unknown mechanism (14). We found that PIASy indeed diminished the ligand-dependent transcriptional activation of wild type AR (Fig. 1A), and in a dose-dependent manner (see Fig.S1. in the supplemental material). We found that this inhibition may be independent of sumoylation as several AR sumoylation site mutants remained sensitive to PIASy-mediated inhibition (Fig. 1A, K386R, K520R, and 2KR mutants).

Like other PIAS family members, PIASy contains a conserved RING finger domain with SUMO-E3 ligase activity. The cross-braced arrangement of the RING domain by conserved cysteine residues is critical for the E3 ligase activity of several different E3

11

enzymes (24). To determine the requirement of RING domain in PIASy-mediated inhibition of AR, we generated substitution mutations by replacing cysteines 347 and 342 with alanines, creating C347A and C342A mutants with disruption of the first and second Zn^{2+}-chelating structures, respectively (Fig. 1B). A luciferase reporter assay showed that the C342A, but not the C347A mutant, reduced the propensity of PIASy to inhibit AR's transcriptional activity (Fig. 1C). These results indicate that the integrity of RING domain is required and the second Zn^{2+}-chelating structure is most crucial in determining PIASy's inhibitory effect on AR.

Given that the integrity of RING domain is necessary for PIASy-mediated repression on AR through a sumoylation-independent pathway, we hypothesized that other ubiquitin-like modification may be involved. To investigate how ubiquitin-like modifications are involved in the PIASy-mediated regulation of AR, Ubiquitin, SUMO-1, ISG15, and NEDD8 were tagged with His_6 epitope and co-expressed with HA-AR and wild-type PIASy in HEK293 cells. Substrates covalently conjugated with each ubiquitin-like modifier were pulled down by Ni^{2+}-NTA agarose beads under strong denaturing conditions and detected by Western blot. Strikingly, instead of ubiquitination, ISGylation, or sumoylation, we found that PIASy catalyzed a substantial neddylation reaction on AR (Fig. 1D). These results suggest that, in the presence of PIASy, AR is modified preferentially by NEDD8.

To evaluate the specificity of PIASy-mediated neddylation of AR, we tested whether Mdm2, a demonstrated E3 ligase for p53 neddylation (32), could possibly stimulate neddylation of AR. Notably, we found that Mdm2 failed to catalyze neddylation of AR under condition where AR is neddylated by PIASy (Fig. 1E). Conversely, PIASy could not catalyze neddylation of p53, while p53 was heavily neddylated in the presence of Mdm2 (Fig. 1F). These results indicate that PIASy catalyzes neddylation of AR in a substrate-specific manner.

The RING Domain is Critical for PIASy-mediated AR Neddylation

To determine the involvement of the RING domain of PIASy in its ability to catalyze neddylation of AR, the wild type and RING domain mutants of PIASy were tagged with HA epitope and co-expressed with His-NEDD8 and HA-AR in HEK293 cells. Ni^{2+}-NTA agarose beads pull down assay revealed that the wild-type PIASy caused strong neddylation of AR as before, as well as self-neddylation of PIASy (Fig. 1G). Strikingly, the C342A mutation completely abrogated the NEDD8-E3 ligase activity of PIASy on both AR and PIASy itself. By contrast, the C347A mutant displayed reduced AR neddylation, and failed completely in self-neddylation. These results suggest that PIASy has auto-neddylation activity and that the second Zn^{2+}-chelating structure of PIASy's RING domain plays a vital role for catalyzing neddylation of AR.

13

Lysine 630 is Essential for AR Neddylation and PIASy-mediated Inhibition

By comparing reported NEDD8 substrates such as Cullins and p53, a similar stretch of lysine residues was found at the hinge region of AR (K630, K632 and K633). In particular, the K630 residue has been shown to be acetylated by CBP and P/CAF (11). To determine whether these lysine residues are involved in AR neddylation, we created K630R and K630R/K632R/K633R (3KR) mutants and analyzed neddylation status of these mutants. Ni^{2+}-NTA agarose beads pull down assay revealed that the amount of neddylated AR was greatly diminished in both mutants (Fig. 2A). As the 3KR mutant retained residual neddylation comparable to that of the K630R mutant, K630 is likely critical for AR neddylation.

Since the K630R and 3KR mutants of AR were defective in neddylation, they offer an opportunity to determine the contribution of neddylation to PIASy-mediated transcriptional inhibition. Provided that PIASy inhibits AR activity through K630 neddylation, then both K630R and 3KR mutants should be resistant to PIASy-mediated inhibition. In a transient transfection assay, we found that indeed both K630R and 3KR mutants were capable of activating reporter expression in response to DHT, yet PIASy could only inhibit the wild-type AR, but not the K630R or 3KR mutant (Fig. 2B). These results support the conclusion that neddylation of AR at distinct lysine residues is the mechanism whereby PIASy inhibits AR's transcriptional activity.

PIASy Inhibits Transcription and Promotes Neddylation of ER and PR

Although the hinge region is less conserved among members of the NR superfamily, a similar stretch of lysine residues also exists in other receptors. Therefore, we determined whether the mechanism of PIASy-mediated neddylation and transcriptional inhibition was applicable to other members of the NR superfamily. Surprisingly, we found that PIASy also catalyze neddylation of ERα (Fig. 2C) and PR-B (Fig. 2D), as well as several other NRs (data not shown). Furthermore, PIASy was capable of inhibiting transcriptional activation by ERα (Fig. 2E) and PR (Fig. 2F), both in a dose-dependent manner. These results suggest that the mechanism of neddylation and transcriptional inhibition by PIASy is applicable to several different NRs.

Jab1 Forms a Stable Complex with AR

Given that AR's transcriptional activity was negatively modulated by PIASy-mediated neddylation, we were intrigued and decided to elucidate the opposing regulatory mechanism by exploring potential deneddylation enzyme. An androgen-stimulated AR complex was purified from CWR22Rv1, a prostate cancer cell line stably expressing S-tagged AR (18). Silver staining of the purified AR complex revealed several protein bands (Fig. 3A, left panel), including an approximately 40-kDa polypeptide. This 40-kDa

15

polypeptide was later identified as the Jun activation domain-binding protein Jab1, as demonstrated by Western blot and showed slight androgen-enhanced association (Fig. 3A, right panel). The association between Jab1 and AR was further investigated by transient co-expression followed by co-immunoprecipitation in the presence of DHT. Four polypeptides of transfected FLAG-Jab1 were precipitated by anti-FLAG monoclonal antibodies, while two HA-AR forms were co-immunoprecipitated and detected by Western blot (Fig. 3B). It appears that the upper AR form was preferentially co-precipitated with FLAG-Jab1 (Fig. 3B). In a reverse experiment, the 47-kDa polypeptide of Jab1 was preferentially precipitated with AR, whereas the 37-kDa polypeptide of Jab1 showed a weaker interaction (Fig. 3C). In the glutathione S-transferase (GST) pull down assay, GST-Jab1 interacted strongly with the full-length AR, and we mapped the AR-interacting domain of Jab1 to its C-terminal domain between amino acid residues 191 and 272 (Fig. 3D and 3E). Additionally, we determined that AR interacts with Jab1 through its ligand-binding domain (LBD) (Fig. 3F). These results indicate that Jab1 physically interacts with AR *in vivo* and *in vitro*.

The De-neddylation Function of Jab1 is Essential for AR Coactivation

The association of Jab1 with an androgen-stimulated AR complex suggests potential involvement of Jab1 in the ligand-dependent transactivation of AR. Thus, we determined the effect of Jab1 on

AR's transcriptional activity. In a transient transfection assay, ectopic expression of Jab1 resulted in a dose-dependent enhancement of reporter gene expression activated by liganded AR in multiple cell lines (Fig. 4A, see also Fig. S2). However, overexpression of NEDP1, another NEDD8-specific protease, barely altered AR's transcriptional activity (Fig. S3), suggesting that Jab1 but not the other deneddylation enzyme may function as a specific coactivator for AR. This coactivator hypothesis was further supported by silencing of endogenous Jab1, which caused a significant reduction in reporter gene expression activated by AR (Fig. 4B). Together, these data suggest that both ectopically expressed and endogenous Jab1 function as coactivators to enhance the transcriptional activity of AR.

We further determined the domains of Jab1 that are required for its coactivator function on AR. Deletion analyses suggested that the C-terminal amino acid 191-334 domain failed to enhance AR's transcriptional activity (Fig. S4), despite that it could interact with AR (Fig. 3E). This result suggests that, in addition to physical interaction, other activity of Jab1 may be also essential for Jab1's coactivator function. Jab1 contains a Jab1/MPN metalloenzyme (JAMM) motif where lies the isopeptidase activity and is a prerequisite for CSN complex-dependent deneddylation pathway (9, 19). To determine whether the isopeptidase activity is involved in Jab1's coactivator function, we generated a substitution mutation in the JAMM motif by replacing the aspartic acid at residue 151 with asparagine to

create the D151N mutant. Identical mutation has been previously shown to successfully assemble into the CSN complex and to abrogate the deneddylation activity of Jab1-containing complex (8, 9). In our assay, the D151N mutant completely lost its coactivator function on AR (Fig. 4C), suggesting that the deneddylation activity of Jab1 is involved in AR coactivation. To exclude the possibility that the inability to enhance AR transcriptional activity was due to loss of interaction with AR, we also proved that both the wild type and D151N mutant of Jab1 co-immunoprecipitated equally well with AR *in vivo* (Fig. S5). Together, these results suggest that, in addition to physical interaction, the isopeptidase activity is also required for the coactivator function of Jab1 on AR.

Silencing of Jab1 Enhances AR Neddylation and Inhibits Cancer Cell Proliferation

Based on the linkage between Jab1's isopeptidase activity and deneddylation pathway, we speculated that deneddylation might act as the molecular mechanism whereby Jab1 enhanced AR activity. Additionally, AR might be a target of the deneddylation pathway since Jab1 directly interacts with AR. To verify this hypothesis, we first immunoprecipitated endogenous NEDD8 conjugated proteins from LNCaP cells with human NEDD8 antiserum and examined whether endogenous AR is among those neddylated proteins by Western blot. Indeed, endogenous AR was modified by NEDD8 and the neddylated AR

species were quickly decreased upon DHT treatment for 30 minutes (Fig. 5A).

Conversely, we used AR specific antibody to pull down endogenous AR proteins and detected those immunoprecipitated species by anti-NEDD8 antibody. Since neddylation is a dynamic process (20), cells with down-regulated expression of deneddylation enzymes such as Jab1 are expected to retain more neddylated proteins. Thus, we stably silenced the expression of endogenous Jab1 in LNCaP prostate cancer cells by lentivirus-mediated RNA interference against Jab1, and analyzed the neddylation status of endogenous AR. As illustrated in Fig. 5B, endogenous Jab1 protein was significantly reduced in Jab1-silenced cells in comparison to cells infected with control virus. The reduction in Jab1 level was not affected by DHT treatment, and the endogenous AR level was not affected by Jab1 silencing. Interestingly, Western blot analysis of immunoprecipitated AR using anti-NEDD8 antibody detected two neddylated species of AR in Jab1-silenced LNCaP cells in the absence of DHT treatment. These results suggest that the neddylated species of AR can be significantly accumulated in Jab1-silenced cells, suggesting that, first, endogenous AR is subject to NEDD8 modification and, second, Jab1 is involved in the deneddylation of neddylated AR. Particularly, neddylated AR was eliminated upon androgen administration for only 30 minutes (Fig. 5B), which is consistent with the result shown in Fig. 5A and correlates well with the observation of an androgen-

19

stimulated transient association between Jab1 and AR in prostate cancer cells (Fig. 5C, upper panel). In addition to Jab1, we found, in this AR complex, another subunit of the CSN complex, CSN1 (Fig. 5C, middle panel), suggesting that the CSN complex is involved in regulating AR's transcriptional activity.

Given that knocking down the expression of endogenous Jab1 diminished AR's transcriptional activity in a reporter assay (Fig. 4B), we were interested in knowing the effect of Jab1 silencing on endogenous AR target gene expression. As shown in Fig. 6A, knockdown of Jab1 significantly reduced the steady state level of endogneous PSA without altering AR protein level. Upon ligand treatment for 24 hours, PSA protein level was significantly induced in LNCaP cells with control shRNA but not in Jab1-silenced cells. These results confirm that Jab1 acts as an essential coactivator for AR target gene activation.

As most prostatic tumor cell growth required AR's transcriptional activity that is diminished upon silencing endogenous Jab1, we were intrigued to evaluate the therapeutic potential of silencing Jab1 in treating prostate cancer. To assess whether Jab1 controls the growth of prostate cancer cells, we infected LNCaP cells with a lentivirus expressing shRNA directed against Jab1. When compared with cells infected by control virus, we found that the proliferation of LNCaP cells was significantly inhibited by Jab1 silencing (Fig. 6B). Likewise, down-regulation of endogenous Jab1 impaired the growth of LNCaP

cells in a soft agar assay (Fig. 6C and 6D). These results support the physiological relevance of Jab1 in the control of androgen-dependent gene expression and prostate cancer cell growth.

DISCUSSION

Our results provide a significant revision of the understanding of androgen-induced transcriptional activation. By exploring the possibility of other ubiquitin-like modifications involved in the PIASy-mediated regulation of AR, we found that AR is modified by NEDD8 (Fig. 1C and 1D). In particular, PIASy catalyzes neddylation of AR and inhibits androgen-dependent transcription (Fig. 1A). PIASy is a member of Siz/PIAS family. The unique characteristic of Siz/PIAS RING (SP-RING) motif is lacking the second and sixth cysteine residues (Fig. 1B), which are part of the first Zn^{2+}-chelating structure in the so-called cross-brace arrangement of canonical RING domain (24). Although the structural conformation remains unresolved, we suspected that the second Zn^{2+}-chelating structure of RING motif plays a major role in NEDD8-E3 ligase function, given that the C342A mutant of PIASy cannot catalyze neddylation of AR and consequently fails to repress AR's activity (Fig. 1C and 1G lane6). Noticeably, another PIAS family member, PIAS3, also contains E3 ligase activity of sumoylation even with only one cysteine in the first Zn^{2+}-chelating structure. These RING domain mutants of PIASy may be useful as tools to decipher the structural and functional

21

relationship in terms of recruiting substrate and whether this phenomenon is conserved among other PIAS family.

Most significantly, we have determined that K630, K632 and K633 within the hinge region of AR are potential neddylation sites. Although those lysine residues are located in a less conserved region, similar stretches of lysine residues (KXKK/RXKK) also exist in other NRs and are phylogenetically conserved among different species. Similar to AR, several NRs including ERα (Fig. 2C and 2E), PR (Fig. 2D and 2F), ERβ, and PXR (data not shown) were all susceptible to PIASy-mediated neddylation and transcriptional inhibition, suggesting that neddylation at those sites may be a prototypic mechanism to modulate NR activity.

In contrast to the inhibitory effect of PIASy, we isolated Jab1 in a transient androgen-stimulated AR complex (Fig. 3A) and shown that Jab1 is capable of enhancing androgen-induced AR's transcriptional activation (Fig. 4A). The essence of JAMM for Jab1's coactivator function on AR prompted us to identify endogenously neddylated AR. Indeed, androgen-induced gene transcription was abolished and neddylated AR was accumulated in LNCaP cells with down-regulation of Jab1 (Fig. 4B and 5B), suggesting that deneddylation activity of Jab1 is the mechanism through which Jab1 enhanced AR's transcriptional activity. Intriguingly, without ligand treatment, the neddylated AR in Jab silencing LNCaP (Fig. 5B, lane 4) demonstrates slower mobility

(MW>175kDa) in a SDS-PAGE than that in parental cells (Fig. 5A, lane 2), indicating poly-neddylation (larger than tetramer) or neddylation at multiple sites will be easier preserved in Jab1 silencing cells. In addition, the involvement of CSN1 in the same active AR complex suggests that the whole CSN complex may be linked to activate androgen-dependent transcription. It remains to be determined whether Jab1 alone is sufficient or the whole CSN complex is required to remove the modification of NEDD88 on AR upon androgen administration. On the other hand, the multiple bands appeared in the overexpression of Jab1 remain mysterious (Fig. 3B). The 47-kDa polypeptide of Jab1 seems to have a post-translational modification since it doesn't appear in *in vitro* transcribed and translated products (data not shown). In a sucrose gradient sedimentation experiment with whole cell lysate of LNCaP cells, we observed both 47-kDa (modified) and 37-kDa (unmodified) forms of Jab1 co-fractioned with AR in a high molecular weight complex (data not shown), which indicates that the 47-kDa polypeptide is a genuinely modified form of Jab1. It would be interesting to know what are the modifications and whether they affect the deneddylation activity of Jab1.

To evaluate the biological significance of AR neddylation, we tested the therapeutic potential of silencing Jab1 for treating prostate cancer by characterizing the growth of LNCaP cells infected with lentivirus containing shRNA against endogenous Jab1. Strikingly, both the proliferation rate and tumor cell growth in a soft agar assay were significantly impaired by Jab1

23

silencing (Fig. <u>6B</u> and <u>6D</u>). The growth impairment could be resulted from the down regulation of AR activity that is required for androgen-dependent prostate cancer cells such as LNCaP (Fig. <u>4B</u> and <u>6A</u>). Also, p53-dependent apoptosis and/or p27-mediated cell cycle arrest may contribute to inhibit cell proliferation as p53 and p27 were accumulated in a Jab1 nullification mouse model (28). These results revealed the physiological relevance of Jab1 in the control of androgen-dependent gene expression and cancer cell growth.

In summary, our data led us to propose a reversible neddylation mechanism achieved by PIASy and Jab1 that stringently regulates AR's transcriptional activity and cancer cell growth. As shown in Fig. 7, we propose that PIASy catalyzes AR neddylation in the absence of ligand. Our previous study showed a specific interaction between AR and silencing mediator for retinoid and thyroid hormone receptor (SMRT), which is substantially enhanced by the presence of hinge region of AR (17). Intriguingly, our current study suggests that the neddylation sites of AR are located in the hinge region. It is tempting to speculate that neddylation at the hinge region may act as an adaptor to stabilize NR's interaction with SMRT and repress transcription in the absence of ligand. Upon ligand treatment, Jab1 as well as other components of CSN complex start to associate with AR, remove NEDD8 modification, and dissociate SMRT from AR. Under this condition, coactivator complexes are allowed to interact with liganded AR and fully activate

24

transcription. It is necessary to understand the detail mechanism whereby neddylation inhibits AR's transcriptional activity. Also, It will be important to systematically characterize the incidence of AR neddylation and expression profiles of Jab1 and PIASy in different progression stages of prostate cancer including androgen dependent and independent diseases to offer new targets for treating prostate cancer.

FOOTNOTES

† Supplemental data for this article may be found at http://mcb.asm.org/.

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FIG. 1. PIASy inhibits AR's transcriptional activity and promotes neddylation of AR in vivo. (A) In a PSA-driven reporter assay, wild type AR and mutants with sumoylation sites mutation (K386R, K520R or 2KR) are all sensitive to PIASy-mediated inhibition in a transient transfection assay in HEK293 cells. (B**)** The cross-braced arrangement of SP-RING structure of PIASy. C347 resides in the first zinc chelating structure while C342 locates in the second one. (C) A luciferase reporter assay in HEK293 cells shows that the C342A, but not the wild type or C347A mutant of PIASy reduces the propensity to inhibit AR's transcriptional activity. (D) A Ni^{2+}-NTA agarose beads pull-down assay shows that PIASy catalyzes significant neddylation of AR instead of ubiquitination, sumoylation, or ISGylation in HEK293 cells. (E) PIASy, but not Mdm2, catalyzes the neddylation of AR in COS7 cells. (F) Mdm2, but not PIASy, promotes neddylation of p53 in COS7 cells. (G) The integrity of RING domain is critical for PIASy-mediated neddylation of AR. Wild type PIASy strongly catalyzes both AR and PIASy-self neddylation in HEK293 cells (lane 5). The C342A mutation abrogated both the AR and PIASy-self neddylation (lane 6), while the AR neddylation activity is moderately preserved in C347A mutant (lane 7).

FIG. 2. Conservation of PIASy-mediated neddylation and transcriptional inhibition among NRs. (A) PIASy-mediated neddylation of wild type AR (lane 5) was greatly diminished in

both K630R and 3KR mutants (lanes 6 and 7). His-ISG15 was used as a negative control (lane 8). The Western blots of ectopic expression levels of AR and PIASy were determined, as well as β-actin as a loading control (lower panel). (B) In a MMTV-driven reporter assay, both K630R and 3KR mutants but not wild type AR were resistant to PIASy-mediated transcriptional inhibition. (C-F) PIASy-mediated neddylation and inhibition is conserved in both ERα and PR. Ni^{2+}-NTA pull down assays show PIASy catalyzed the neddylation of ERα (C) and PR-B (D). PIASy-mediated inhibitions on the transcriptional activities of ERα (E) and PR-B (F) were determined by measuring pS2 and MMTV promoter-driven luciferase gene expression In HEK293 cells, respectively.

FIG. 3. Jab1 is a component of the AR complex in prostate cancer cells. (A) CWR22Rv1 prostate cancer cells were stably transfected with S-tag hAR, and treated with solvent (ethanol) or DHT (10 nM for 10 min). AR complexes were purified using S protein column, resolved on SDS-PAGE and visualized by silver staining (left panel). The AR protein and an unknown protein around 40-kDa were marked. This 40-kDa protein was identified as Jab1 by Western blot using goat anti-Jab1 antibody (right panel). (B and C) AR interacts with Jab1 *in vivo.* Immunoprecipitation was conducted using mouse anti-FLAG (FG) (B) and mouse anti-HA (C) monoclonal antibodies, respectively. The IP products were analyzed by Western blot (WB) with indicated antibodies. (D) The schematic map of GST fused full-length as well as different

fragments Jab1 proteins is shown. (E) GST pull-down assay maps the AR interaction domain of Jab1 to aa 191-272 (marked as AR-ID in (D)). (F) GST pull-down assay shows the AR LBD, but not AB or DBD region, interacted with GST-Jab1 full-length and the aa 191-272 fragment.

FIG. 4. The Jab1/MPN metalloenzyme (JAMM) motif is essential for the coactivation function of Jab1. (A) In a luciferase reporter assay, ligand-dependent AR activation is enhanced by ectopic expression of wild type Jab1 in a dose-dependent manner in HEK293 cells. (B) HEK293 cells were infected by lentivirus containing shRNA against Jab1. Ligand induced AR transcriptional activity is blocked by silencing endogenous Jab1. (C) Jab1's enhancement activity is dependent on JAMM motif. In HEK293 cells as overexpression of JAMM motif mutant (D151N) failed to enhance ligand-induced PSA-driven luciferase expression. The protein expression levels of AR and Jab1, as well as the loading control β-actin were also determined (lower panels in A-C).

FIG. 5. Endogenous AR is modified by NEDD8. (A) Neddylation of endogenous AR. LNCaP cells were maintained in stripped media for at least four days and endogenous neddylated AR was detected by immunoprecipitating neddylated proteins with mouse anti-NEDD8 antibody and immunoblotted by mouse anti-AR antibody. (B) Neddylation of endogenous AR is enhanced in Jab1 knockdown cells. In LNCaP cells with Jab1 silencing, IP was conducted by mouse anti-AR antibody and detected by rabbit anti-NEDD8 antibody

(arrow). Knockdown of endogenous Jab1 was verified by Western blotting. β-actin serves as a loading control. (C) Association of Jab1 and CSN1 with AR upon DHT treatment. CWR22Rv1 prostate cancer cells stably transfected with S-tag hAR were treated with solvent (ethanol) or DHT (10 nM) for indicated time period. AR complexes were purified using S protein column, resolved on SDS-PAGE and detected by goat anti-Jab1 or rabbit anti-CSN1 antibodies, respectively.

FIG. 6. Effects of Jab1 Silencing on Prostate Cancer Cell Growth. (A) In LNCaP cells, silencing Jab1 abolishes ligand-induced expression of endogenous AR targeting gene, PSA. (B) Silencing of endogenous Jab1 inhibits LNCaP cell proliferation. The proliferation curves were determined for 6 days from three independent experiments in triplicate. (C and D) Knockdown of endogenous Jab1 impaired LNCaP cell grwoth in a soft agar assay. Photographs show a representative pair of LNCaP cell colonies formed on soft agar of the control and Jab1 silenced cells (C). The average colony numbers were determined from three independent experiments in triplicate (D). The number of colonies formed on the soft agar in the Jab1 silenced cells is signicantly less (p<0.01).

FIG. 7. A working model of modulation of AR's transcriptional activity by reversible NEDD8 modification. Without ligand, PIASy catalyzes AR neddylation in the hinge region which stablize the AR's interaction with corepressor complex (CoR) and efficiently

34

repress transctiprion. Upon ligand treatment, Jab1 as well as other components of CSN complex interact with AR, remove NEDD8 modification, and dissociate CoR complex. Under this condition, the coactivator complex (CoA) is allowed to interact with AR and fully activate transcription.
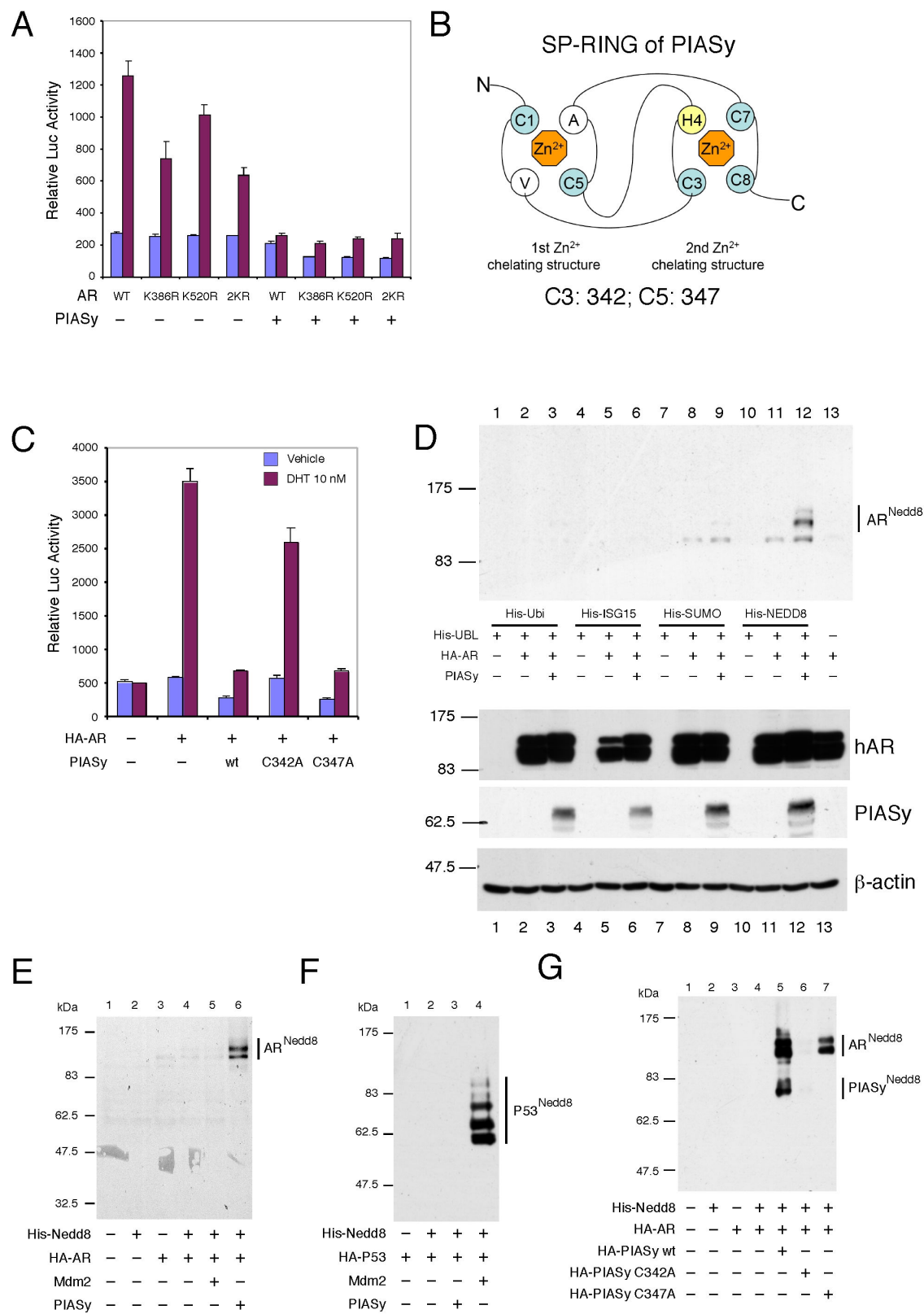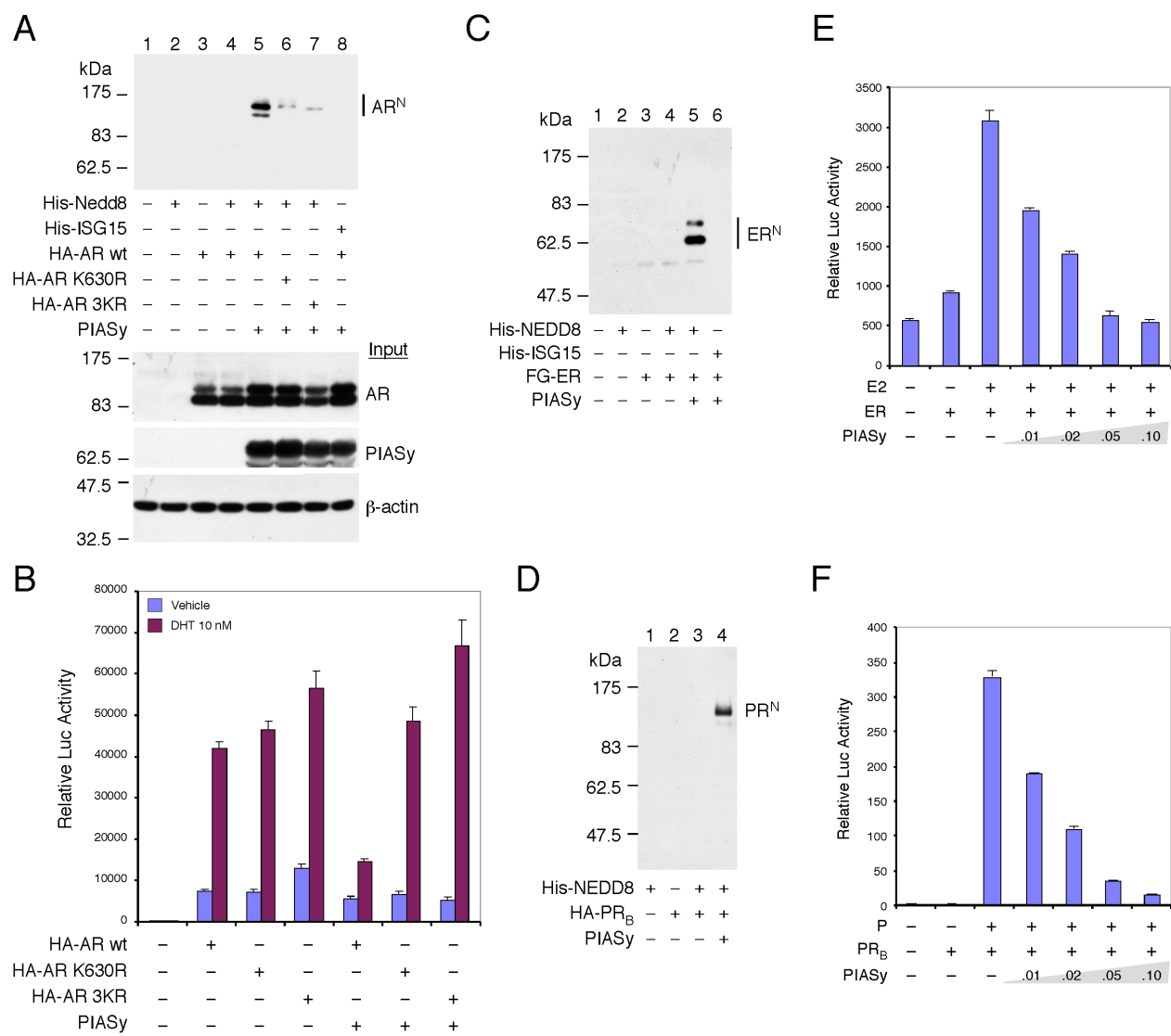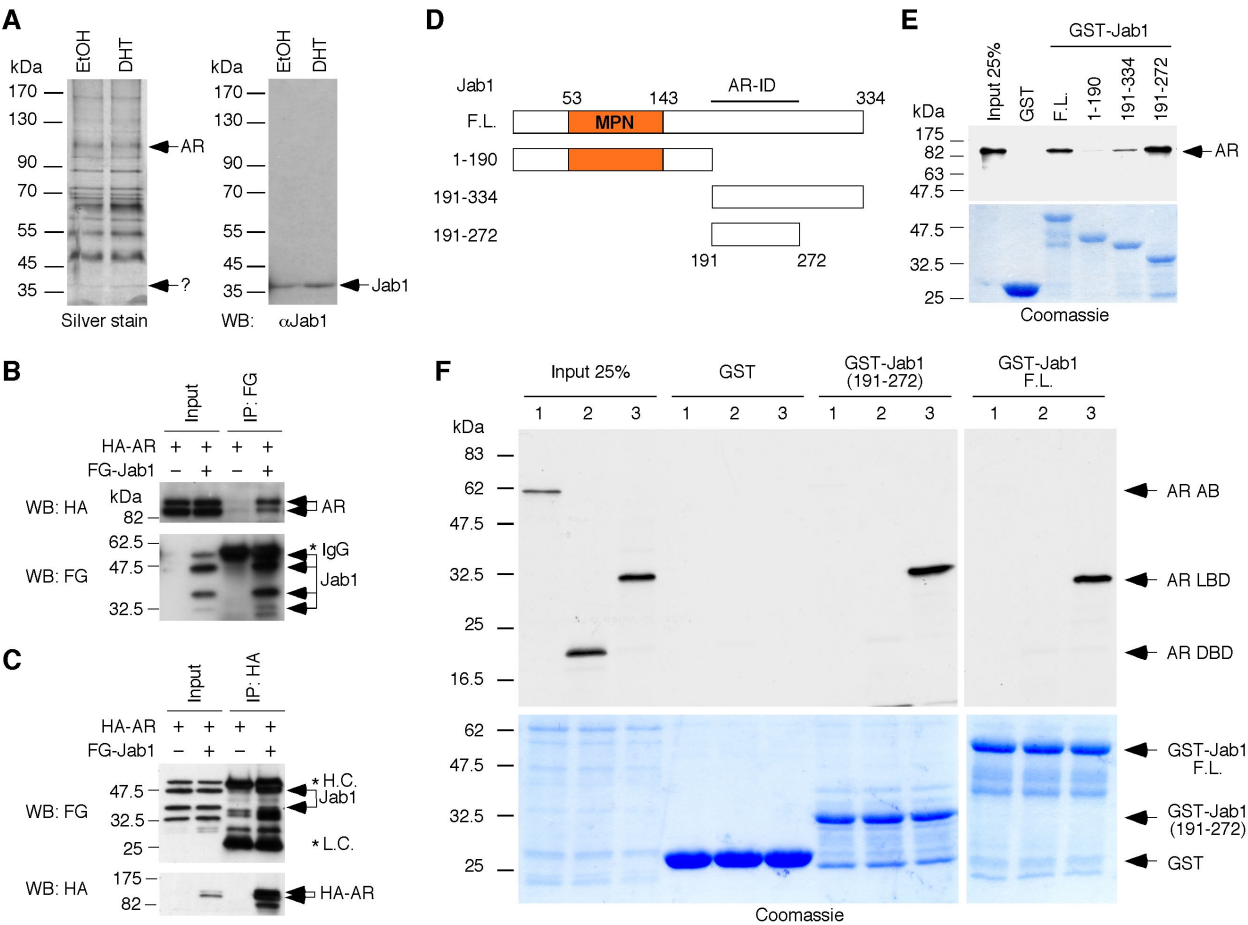
Figure 1

A

B SP-RING of PIASy

1st Zn^{2+} chelating structure   2nd Zn^{2+} chelating structure

C3: 342; C5: 347

C

D

AR^{Nedd8}

	His-Ubi			His-ISG15			His-SUMO			His-NEDD8		
His-UBL	+	+	+	+	+	+	+	+	+	+	+	+	–
HA-AR	–	+	+	–	+	+	–	+	+	–	+	+	+
PIASy	–	–	+	–	–	+	–	–	+	–	–	+	–

hAR

PIASy

β-actin

E

AR^{Nedd8}

	1	2	3	4	5	6
His-Nedd8	–	+	–	+	+	+
HA-AR	–	–	+	+	+	+
Mdm2	–	–	–	–	+	–
PIASy	–	–	–	–	–	+

F

P53^{Nedd8}

	1	2	3	4
His-Nedd8	–	+	+	+
HA-P53	+	+	+	+
Mdm2	–	–	–	+
PIASy	–	–	+	–

G

AR^{Nedd8}

PIASy^{Nedd8}

	1	2	3	4	5	6	7
His-Nedd8	–	+	+	+	+	+	+
HA-AR	–	–	+	+	+	+	+
HA-PIASy wt	–	–	–	–	+	–	–
HA-PIASy C342A	–	–	–	–	–	+	–
HA-PIASy C347A	–	–	–	–	–	–	+

36

Figure 2

Figure 3

Figure 4

Figure 5

A

DHT   –   +   (30 min)

IP: m α hNEDD8
IB: m α hAR

IgG   NEDD8   IgG   NEDD8

175 —

} AR^{Nedd8}

83 —

DHT   –   +   input

AR

B

siRNA   Cont.   Jab1
DHT   +   –   +   –   (30 min)

Jab1

β-actin

kDa

AR

83 —

175 —

} AR^{Nedd8}

83 —

C

Vehicle   DHT 10nM
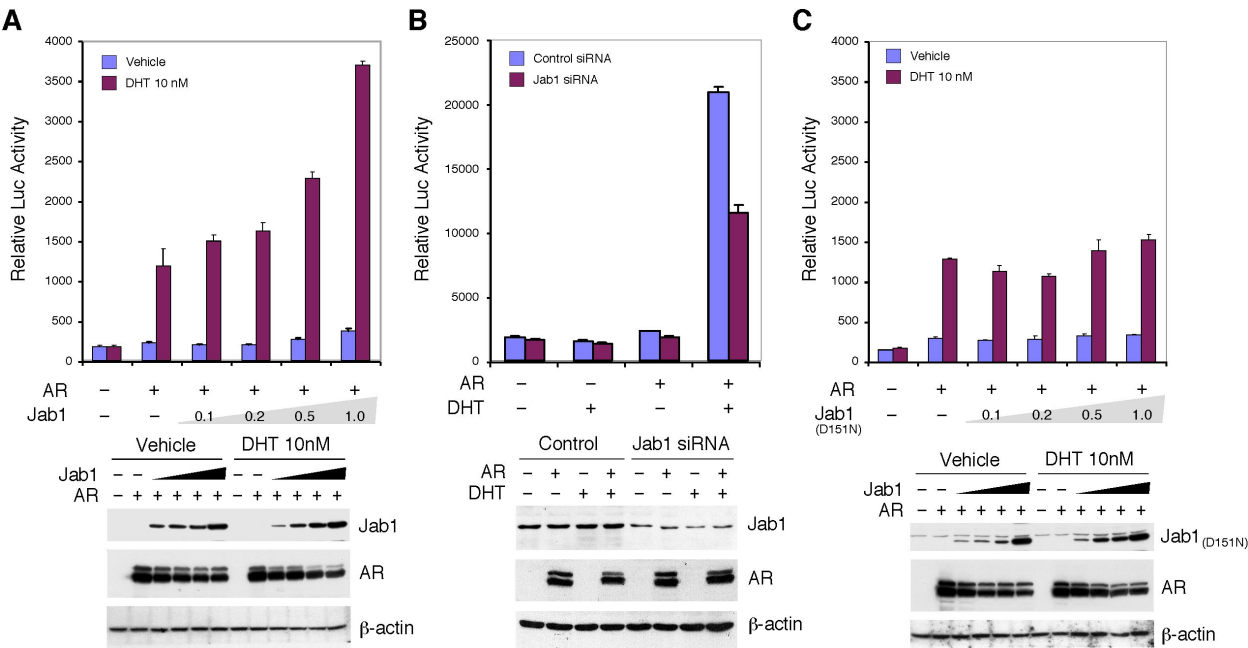10   5   10   15   30   (min)

Jab1

CSN1

AR
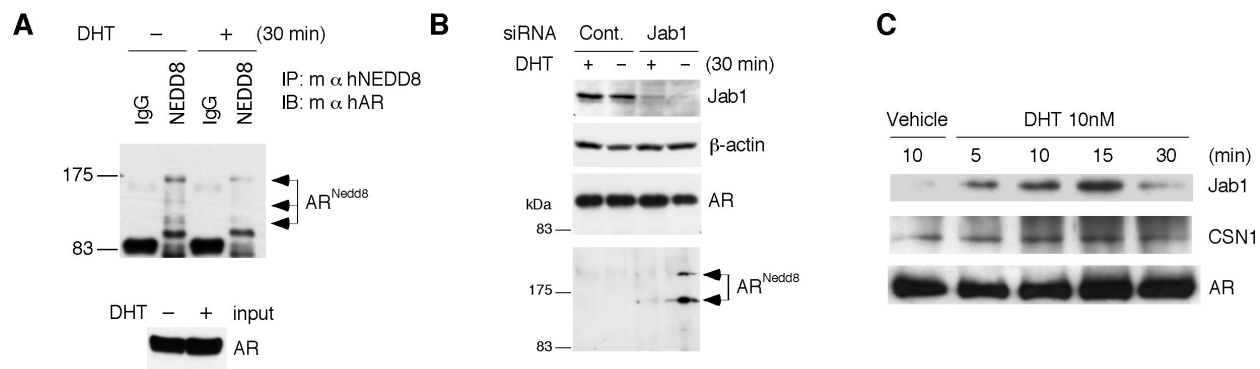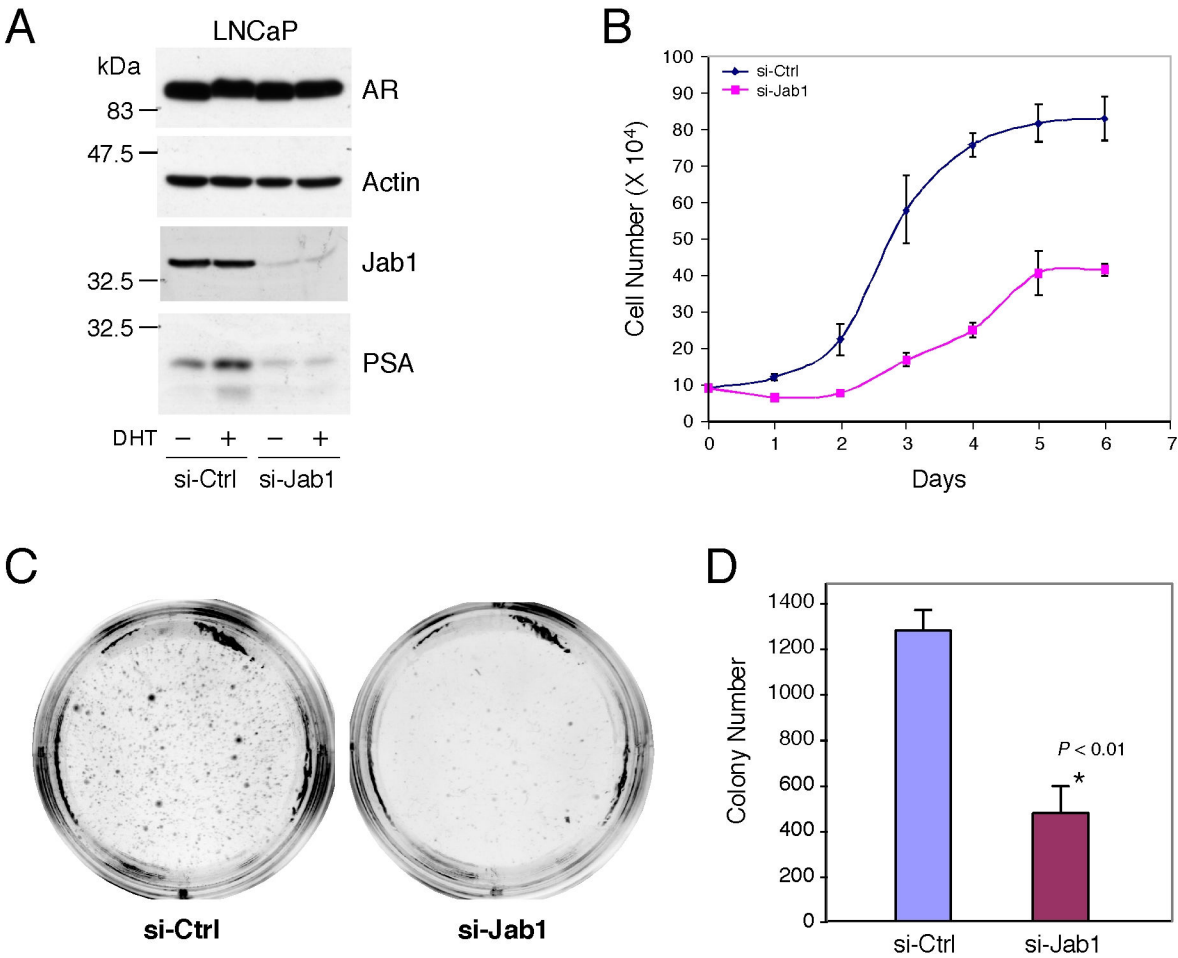
Figure 6

Figure 7
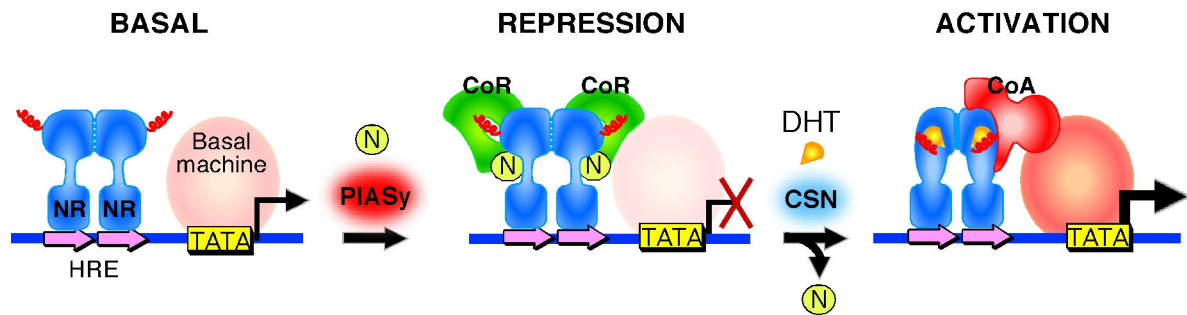
Appendix 2.

Li, C.-W., Ai, N., Dinh, G. K., Welsh, W. J. and Chen, J. D. (2010) Human ADA3 Regulates RARα Transcriptional Activity through Direct Contact between LxxLL Motifs and the Receptor Coactivator Pocket. *Nucl. Acid Re*search. 38: 5291-303.

Human ADA3 regulates RARα transcriptional activity through direct contact between LxxLL motifs and the receptor coactivator pocket

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ABSTRACT

The alternation/deficiency in activation-3 (ADA3) is an essential component of the human p300/CBP-associated factor (PCAF) and yeast Spt-Ada-Gcn5-acetyltransferase (SAGA) histone acetyltransferase complexes. These complexes facilitate transactivation of target genes by association with transcription factors and modification of local chromatin structure. It is known that the yeast ADA3 is required for nuclear receptor (NR)-mediated transactivation in yeast cells; however, the role of mammalian ADA3 in NR signaling remains elusive. In this study, we have investigated how the human (h) ADA3 regulates retinoic acid receptor (RAR) α-mediated transactivation. We show that hADA3 interacts directly with RARα in a hormone-dependent manner and this interaction contributes to RARα transactivation. Intriguingly, this interaction involves classical LxxLL motifs in hADA3, as demonstrated by both 'loss' and 'gain' of function mutations, as well as a functional coactivator pocket of the receptor. Additionally, we show that hADA3 associates with RARα target gene promoter in a hormone-dependent manner and ADA3 knockdown impairs RARβ2 expression. Furthermore, a structural model was established to illustrate an interaction network within the ADA3/RARα complex. These results suggest that hADA3 is a _bona fide_ transcriptional coactivator for RARα, acting through a conserved mechanism involving direct contacts between NR boxes and the receptor's co-activator pocket.

INTRODUCTION

Retinoic acids (RAs), the oxidized forms of vitamin A, possess important physiological and pharmacological actions as they determine vertebrae development, promote cell differentiation and affect cancer cell proliferation (1). The genomic actions of RAs are mediated by RA receptors (RARs), members of the nuclear receptor (NR) superfamily. NRs are ligand-dependent transcription factors that control gene expression in target cells. To date, many NRs are known as targets for therapeutic drugs in treating various human disorders, including metabolic diseases and cancers (2). Structurally, NRs share a common domain organization that consists of a variable N-terminal activation function domain (AF-1), a central DNA-binding domain (DBD) followed by a short hinge/D region, and a C-terminal ligand-binding and ligand-dependent transactivation function domain (LBD/AF-2) (3). The highly conserved DBD contains two zinc fingers and is responsible for recognition of hormone responsive elements (HREs). The LBD contributes to a dimerization interface of the receptor, in addition to binding co-activators and corepressors.

The transcriptional activity of NRs is thought to be regulated by a dynamic exchange of co-activators and corepressors (4). Crystallographic studies have revealed that ligand-binding induces a conformational change of the LBD and causes a positional shift of the carboxyl-terminal AF-2 helix (helix 12 or H12) (5). This alternation changes the cofactor-binding surface, triggers the dissociation of corepressors such as silencing mediator for retinoid and thyroid hormone receptors (SMRT) and N-CoR (6,7), and subsequently recruits co-activators like p160/SRC and CBP/p300 (8). The p160 co-activator family includes SRC1/NCoA-1 (9), TIF2/GRIP1 (10,11) and pCIP/AIB1/RAC3/ACTR (12,13). These three

conserved co-activators interact with receptors in a hormone- and AF2-dependent manner and facilitate transcriptional activation by NRs. In contrast to the corepressors, co-activators contain intrinsic histone acetyltransferase activity (HAT) and remodel chromatin structure in an ATP-dependent manner, as well as causing covalent modification of histone tails (14). Both corepressors and co-activators interact with the same cofactor-binding pocket on the receptor through their α-helical LxxLL or related motifs (L represents leucine, x represents any amino acids). These LxxLL motifs are also known as CoRNR boxes in corepressors and as NR boxes in co-activators, respectively (15,16). In general, the LxxLL motif adopts a short α-helical structure and docks into a hydrophobic co-activator-binding pocket surrounded by helices H3, H4, H5 and H12 of the LBD (17). Mutational analyses of the co-activators NR boxes have also uncovered a receptor specific code of interaction, suggesting that NR box-mediated differential contacts may determine specific modes of NR action (5).

The alternation/deficiency in activation-3 (ADA3) is an essential component of the human PCAF and yeast SAGA or ADA HAT complexes. These complexes are known to stimulate transcription by association with DNA-binding factors and by modifying local chromatin structure (18,19). Through direct interactions, ADA3 participates in diverse physiological processes, and regulates the functions of several important proteins such as the tumor suppressor p53 (20), HPV E6 (21), several NRs (22) and ANCO-1 (23). In particular, the yeast ADA3 (yADA3) directly interacts with hRXRα and hERα in a ligand-dependent manner to augment their transactivation in yeast cells (24). In contrast, other studies have reported that the mouse ADA3 (mADA3) does not interact with these NRs (25) and the yADA3 and hADA3 reportedly fail to interact with hRARα (24,26). Hence, it is assumed that the hADA3 plays an indirect role in regulating RAR's transcriptional activity through interaction with either RXRs or p160 co-activators (27).

Previously, we have determined the molecular mechanisms of the assembly of RXRα with corepressor and co-activator complexes (28,29) complexes. To extend our understanding of the mechanisms of retinoid signaling, we have characterized the potential physical and functional interactions between hADA3 and hRARα. Our results suggest that hADA3 is essential for hRARα transactivation and that hADA3 interacts directly with hRARα. A favorable structural model of the ADA3 LxxLL motif/RARα complex is established and the underlying molecular interactions are postulated.

MATERIALS AND METHODS

Chemicals and reagents

All-trans RA (atRA), rifampicin (Rif), 1,25-dihydroxyvitamin D3, 17β-estradiol (E2), and 3,5,3′-triiodothyronine (T3) were purchased from Sigma (St. Louis, MO, USA). The anti-ADA3 polyclonal antibody was a generous gift from Dr Pierre Chambon (IGBMC, Illkirch, France). The anti-Actin monoclonal antibody was purchased from Sigma.

Plasmids

The pCMX-hRARα wild-type and E412K mutant, and the pDR5-tk-luc reporter have been described previously (29). The hRARα K399E, ME400/1AA, I402A, PG403/4AA, SM405/6AA, PP407/8DT, LI409/10AA, L409A, I410A, Q411A, ML413/4AA, M413A, L414A, E415K, NS416/7AA, EG418/9AA and LD420/1AA mutants were created on the pCMXHA-hRARα template using the Quick Change Site-directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). The series of GAL4 DBD-hRARα H12 mutants for expression in yeast cells were constructed in the pGBT9 vector at NcoI and BamHI cloning sites. The pACT-hADA3, pACT-hADA2α, pACT-hADA2β, pGBT-ANCO-1C (aa 2369–2663) and pGEX-hADA3 are as described previously (23). The NR box motifs of hADA3 were mutated as indicated and the primer sequences (5′ to 3′) for each NR box mutants are: mNR1, CTG CAG CTG GAG GCG GAG ACC GCG GCG TCT TCT GCC; mNR2, AGG TCC GCA CAG CTG AGG AGG CAG CGA AGC CCC CA; mNR3, GAC ACT AAA GAT GCG GCT GCC GCG GCG AAG AAG TCT GAG; mNR4, CTG ACG CAG CGC GCC GCG CAG GCC GCG GCG GAG GAA AAT; mNR5, GAG CGT GAG AGC GCC GCG AAG GCG GCG GAT GGG TAG; V237L, GGA CAC TAA AGA TCT GGA TGC CCT GCT GAA G; V269L, CCT GCA GGC CCT GCT GGA GGA AAA TAT TAT TTC C; I426L, GGA GCG TGA GAG CCT CCT GAA GCT GCT GGA TGG. All constructs have been confirmed by enzyme digestion and DNA sequencing with additional information available upon request.

Yeast two-hybrid assay

Yeast Y190 cells were co-transformed with the GAL4 DBD fusion plasmids (pAS-hRARα, pAS-hRARγ, pGBT-hRARα, pAS-hTRα, pAS-hERα, pGBT-VDR or pGBT-PXR) and the GAL4 AD fusion plasmids (pACT2 vector or pACT-hADA3), together with 4 μg of single-strand salmon sperm DNA according to manufacturer's instructions (Clontech, Palo Alto, CA, USA). Yeast transformants were spread onto synthetic complete plates lacking tryptophan and leucine (SC-Trp-Leu) and incubated for 3 days at 30°C. Colonies were picked and grown in selection media for additional 24 h at 30°C. Aliquots of 100 μl from each sample were taken and added to fresh selection media. For each sample, one aliquot received cognate ligand (atRA, E2 or T3) and the other aliquot received vehicle control (DMSO). After 12 h, yeast cells were harvested and resuspended in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) and permeated with 0.05% SDS. An aliquot of 100 μl Z buffer containing 4 mg/ml ONPG (o-nitrophenyl β-D-galactopyronoside) was added and incubated at 30°C until the appearance of yellowish color. The reactions were stopped by adding 100 μl of 1 M Na_2CO_3 and the incubation time

was recorded. The samples were centrifuged and the OD420 values of the supernatants were measured. The β-galactosidase unit was calculated according to the OD420 value, cell numbers and incubation time.

GST pull-down assay

GST and GST-hADA3 fusion proteins were expressed in *Escherichia coli* BL-21 cells and purified by glutathione agarose beads (GE Healthcare Biosciences, Uppsala, Sweden). Human RARα, ERα and AR were *in vitro* translated and radiolabeled with [^{35}S]-methionine using the TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA). For the GST pull-down experiment, 5 μg of bead-conjugated fusion protein was incubated with 4 μl of *in vitro* translated ^{35}S-labeled protein with moderate shaking at 4°C overnight as previously described (28). Bound proteins were washed three times with fresh binding buffer containing 0.1% NP40, and beads were collected and subjected to SDS–PAGE electrophoresis. Gels were then stained with Coomassie Blue, dried and detected by autoradiography.

Cell culture and transient transfection reporter assay

HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. One day prior to transfection, cells were seeded in 12-well plates at 50 000 cells per well in phenol red free Dulbecco's modified Eagle's medium supplemented with 10% charcoal resin-stripped fetal bovine serum. Transfection was performed using standard calcium/phosphate method as described (29). After transfection, cells were washed with phosphate-buffered saline and re-fed with fresh medium containing vehicle alone or vehicle plus 50 nM atRA and incubated for an additional 48 h. Cells were then harvested and analyzed for luciferase and β-galactosidase activities.

RNA interference

The effect of hADA3 short hairpin RNA (shRNA) on RARα transcriptional activity was analyzed in HEK293 cells. The hADA3, PIASy and a control shRNAs were expressed from the pLL3.7 lentiviral vector (30). The sequence for the hADA3 shRNA is 5′-GAT GAG GCT GAG CAT TAC A-3′ starting at nucleotide 543/amino acid 181 of hADA3 (NM_006354). The sequence for PIASy shRNA is 5′-GCT CTA CGG AAA GTA CTT A-3′ starting at nucleotide 330/amino acid 110 of the human PIASy (BC029874). The control shRNA sequence is 5′-GGT CCG GCT CCC CCA AAT G-3′, which has no homologous sequences in the vertebrate transcriptome. After 24 h of transfection with the shRNA expression plasmid, the HEK293 cells containing transiently transfected hRARα and the luciferase reporter gene were treated with 50 nM atRA for 12 h and analyzed for luciferase activity.

Generation of ADA3 stable knockdown cells

Recombinant lentiviruses were produced by co-transfecting HEK 293T cells with the lentivirus expression plasmid and lentiviral packaging constructs pLP1, pLP2 and the pLP/VSV-G plasmids using Lipofectamine 2000 (Invitrogen). Infectious lentiviruses were harvested at 48 h post-transfection from the culture supernatants, centrifuged to eliminate cell debris and then filtered through 0.22-μm filters. For transducing lentiviral constructs, 50% confluent MCF7 cells were fed with virus containing medium for 2 days to generate 40% infected MCF7 cells by visualizing GFP expression. The top 20% of GFP positive cells were subsequently collected using fluorescence-activated cell sorting analysis to retrieve the highest GFP expressed cells. The lentivirus-infected MCF-7 cells with siCTRL and siADA3 were grown in serum free DMEM for overnight and treated with or without 2 μM atRA for 3 h. Total RNA was extracted using Trizol reagents (Invitrogen), and 2 μg RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) with random oligo(dT) primers. RT products were amplified by PCR using the following primer pairs: for the RARβ2 coding sequence (421 bp), forward, 5′-GGA ACG CAT TCG GAA GGC TT-3′ and reverse, 5′-AGC ACT TCT GGA GTC GAC AG-3′; for the hADA3 coding sequence, forward, 5′-TGT GCC GCA TAC TAA GTC C-3′ and reverse, 5′-GCT TCA GGA TGC TCT CAC GCT-3′; for the control GAPDH coding sequence (212 bp), forward, 5′-GTG GAT ATT GTT GCC ATC A-3′ and reverse, 5′-GAC TCC ACG ACG TAC TCA-3′. PCR products were then separated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining.

Chromatin immunoprecipitation assay

HEK293 cells were transiently transfected with FLAG-tagged hRARα and HA-tagged hADA3 according to the manufacturer's instruction (Lipofectamin 2000, Invitrogen). After treated with 2 μM atRA for 3 h, cells were incubated with 1% formaldehyde in culture media for 10 min at 37°C. The formaldehyde cross-linked cells were washed twice with ice-cold PBS and lysed in SDS buffer [1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.1 and protease inhibitor cocktail (Roche)]. Nuclei were collected, resuspended in lysis buffer and sonicated 10 times with 15-s pulses on ice. Supernatant were then diluted in 10-fold Dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 16.7 mM NaCl) and pre-cleared with 80 μl of salmon sperm DNA-protein G-agarose (Upstate) for 2 h at 4°C. The sheared DNA mixture was subjected to overnight incubation with 1 μg of anti-FLAG antibody (F3165, Sigma), anti-HA antibody (1815015, Roche), anti-RNA polymerase II antibody (05-623B, Upstate) or an equal amount of normal mouse immunoglobulin G (Santa Cruz Biotechnology) at 4°C. Immunoprecipitation was performed using protein G agarose for 1 h at 4°C. The protein/DNA cross-links were reversed by heating at 65°C for 6 h, and then the DNA were extracted using QIAquick PCR purification kit (Qiagen). For PCR reaction, 3 μl from a 40 μl DNA preparation was used for amplifications. The DNA product was analyzed using a primer set against RARβ2 promoter, 5′-AAG

CTC TGT GAG AAT CCT G-3′ and 5′-GGA TCC TAC CCC GAC GGT G-3′ (GenBank accession number AF275948). The 228 bp RARβ2-promoter were visualized on a 1.5% agarose gel.

Molecular modeling

The initial model for RARα/hADA3 complex was constructed based on the crystal structure of the RARβ/RXRα complex with an LxxLL peptide from the TRAP220 co-activator (PDB: IXDK) (31). Due to the high sequence identity shared between RARα and RARβ (~88%), the distinct residues on RARβ were directly mutated to the respective residues on hRARα and the coordinates of the NR box 4 peptide of hADA3 were built from those of the TRAP220 peptide. The resulting models were subjected to energy minimization for 2500 steps of steepest descent geometry optimization followed by 2500 steps of conjugate gradient optimization with RARα LBD held fixed, except for the residues making contact with the ADA3 peptide. The energy minimized structural model of the RARα/ADA3 complex was then utilized as the initial structure for Molecular Dynamics (MD) simulation. The model was first solvated by explicit water molecules, followed by 5000 steps energy minimization. The temperature of the system was heated gradually from 100 to 300 K for 200 ps and kept constant throughout the later simulation. Furthermore, a 500 ps equilibration step was conducted at 300 K and 1 atmosphere constant pressure to adjust the solvent density. Finally, another 2 ns MD simulation was run for data collection with snapshots obtained every 2 ps.

RESULTS

Human ADA3 enhances the transcriptional activity of hRARα in mammalian cells

To investigate the role of hADA3 in RAR signaling, we measured the effects of hADA3 overexpression and silencing on hRARα-mediated transcriptional activation, using a transient transfection reporter assay in the human embryonic kidney HEK293 cells. As shown in Figure 1A, ectopically expressed hADA3 enhanced the activation of RARα-driven transcription in the presence of atRA in an ADA3 concentration-dependent manner, suggesting that hADA3 may function as a co-activator for RARα. To determine whether endogenous hADA3 contributes to the RARα-mediated transcriptional activation, we knocked down the endogenous ADA3 in HEK293 cells by shRNA using a lentivirus vector (Figure 1B, inset). While the control shRNAs had no effect, silencing of hADA3 significantly reduced RARα-mediated transactivation (Figure 1B). Therefore, both the endogenous and ectopically expressed hADA3 are involved in enhancing RARα-mediated transcriptional activation. Additionally, we examined the effect of ADA3 knockdown on the RARα downstream gene regulation. ADA3 knockdown cells were created using lentivirus-based siRNA system in MCF7 cells. As shown in Figure 1C, the expression of RARβ2, upon its induction by atRA, was much lower in the ADA3 knockdown cells as compared with

control cells. This result suggests that hADA3 acts as an RARα co-activator regulating its target gene expression.

ADA3 interacts directly with liganded RARα

Next, we tested if hADA3 interacts directly with RARα. Yeast Y190 cells expressing both the GAL4 DBD-hRARα and GAL4 AD-hADA3 fusions proliferated well on a -Trp-Leu-His+3AT (3-aminotriazole) plate in the presence of atRA (Figure 2A). In contrast, cells without the hRARα or hADA3 fusions were unable to grow under the same conditions regardless of atRA. As controls, all transformants proliferated equally well on -Trp-Leu plates (left panels). This ADA3/RARα interaction was confirmed by using different GAL4 DBD-RAR constructs (Figure 2B), suggesting that hADA3 interacts specifically with RARs in a ligand-dependent manner.

We next examined ADA family proteins, including hADA3, hADA2α and hADA2β, for their interaction potentials with various NRs. Interestingly, only hADA3 was able to interact with these NRs in the presence of cognate ligands, while hADA2α/β show little interaction regardless of ligands (Figure 2C). These results suggest hADA3 subunit as a potential mediator in the recruitment of PCAF/ADA complex to ligand-activated NRs. The interactions between hADA3 and NRs were further demonstrated *in vitro* using GST pull-down assays (Figure 2D), where hRARα and hERα showed specific ligand-enhanced interactions with hADA3 while hAR exhibited a ligand-independent interaction. These results suggest that hADA3 interacts directly with several NRs in a hormone-dependent manner *in vivo* and *in vitro*.

ADA3 binds to the co-activator pocket of RARα

The H12 helix at the carboxyl-terminal end of NR plays a critical role in the formation of a ligand-induced co-activator-binding pocket (17). To determine if the co-activator pocket is responsible for ADA3 binding, we systematically mutated each amino acid spanning the H12 helix of hRARα (Figure 3A). These RARα mutants were labeled with [35S]-methionine and tested by GST pull-down assay for interaction with GST-hADA3 in the presence of atRA *in vitro*. As shown in Figure 3B, SM405/6AA, PP407/8DT, LI409/10AA, L409A, I410A, E412K and ML413/4AA mutations all interrupted RARα interaction with hADA3, suggesting that H12 helix is essential for ADA3 binding. In contrast, the Q411A mutant within the H12 helix did not affect the interaction, suggesting that the side chain of Q411 is not critical for ADA3 interaction.

To compare the RARα interaction profiles between ADA3 and p160 co-activator, yeast two-hybrid assays were carried out to determine the interaction of RARα H12 mutants with ADA3 and RAC3 (Figure 3C). We found that the ligand-induced interaction patterns of hRARα and hADA3 in yeast cells were consistent with that of the GST pull-down assay. Moreover, the hRARα interacting profiles with hADA3 and RAC3 were similar (Figure 3C). As a control, except for the PG403/4AA mutant, none of these GAL4 DBD-fused hRARα mutants displayed significant background activation,
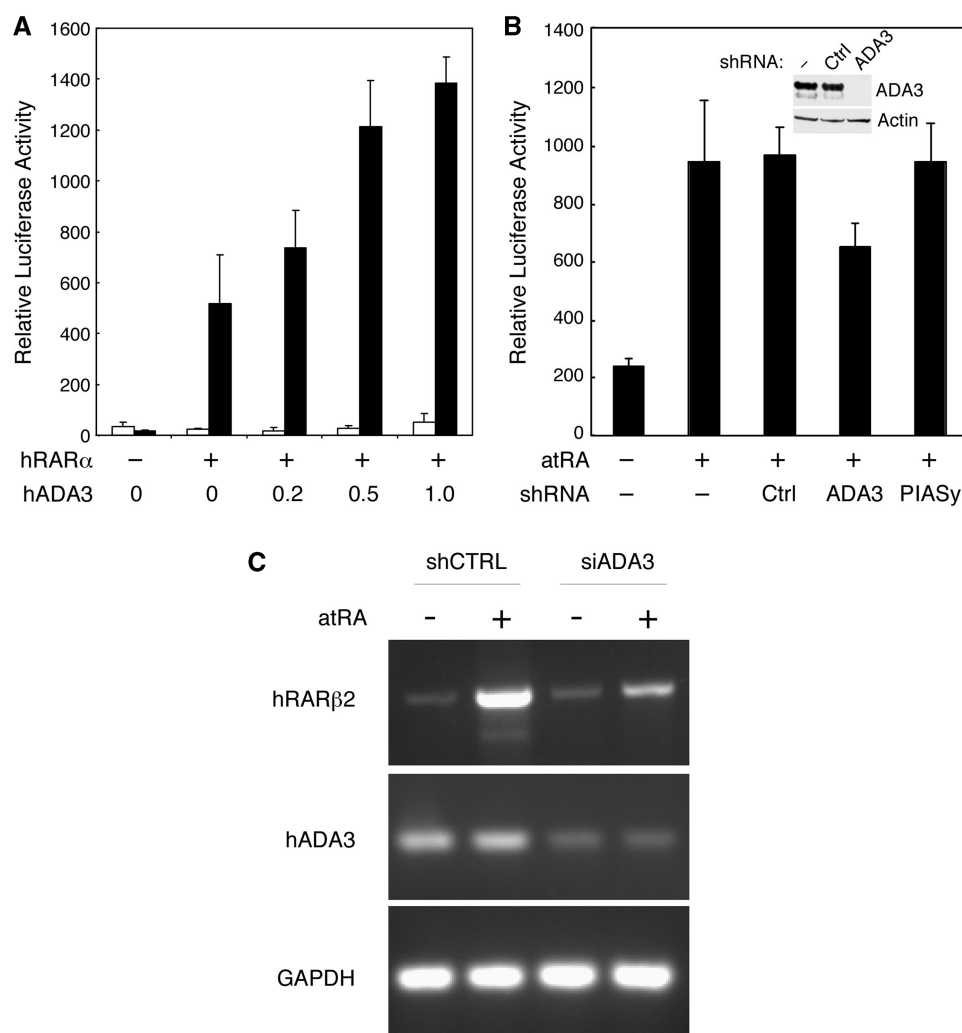
Figure 1. The human ADA3 enhances transcriptional activity of RARα in mammalian cells. (**A**) Ectopic overexpression of human ADA3 enhances transcriptional activation mediated by hRARα. HEK293 cells were cotransfected with pCMX-hRARα, pDR5-tk-Luc reporter and indicated amounts (in μg) of pCMX-hADA3. Cells were incubated in the absence (white bars) or presence (black bars) of 50 nM atRA. The relative luciferase activities were determined from three independent samples after normalization to the internal β-galactosidase control. (**B**) Endogenous human ADA3 contributes to RARα-mediated transcriptional activation. HEK293 cells were cotransfected with pCMX-hRARα and pDR5-tk-Luc reporter plus pLL3.7 shRNA expression vector without an insert (−), or with the control (Ctrl), hADA3 or PIASy targeting shRNA. The relative luciferase activities were determined from three independent samples after normalization to the internal β-galactosidase control. The small inset in the graph is a western blot showing the reduction of ADA3 by the pLL3.7-hADA3 shRNA, which has no effect on the expression of endogenous β-actin. (**C**) Expression of RARβ2 is impaired in ADA3 knockdown cells. Total RNA was extracted from each of the control (siCTRL) or siADA3-infected stable clones without or with atRA treatment and the RNA was subjected to RT-PCR using primers specific for RARβ2 or ADA3 coding sequence. GAPDH was used as an internal control.

(Figure 3C, +pACT2 panel). The H12 requirement and similar interacting profile to RAC3 suggest that hADA3 interacts with the co-activator-binding pocket of hRARα.

ADA3 interaction mutants of RARα display impaired transcriptional activity

To strengthen the idea that ADA3 is a transcriptional co-activator for RARα through a mechanism involving direct protein–protein contact, we assessed the transcriptional potentials of the RARα H12 mutants by transient transfection reporter assay. Since endogenous ADA3 may function as a co-activator for RARα, this experiment was

conducted in the absence of ADA3 overexpression. As shown in Figure 4, the RARα H12 mutants that are defective in ADA3 interaction also displayed impaired transactivation function. These data suggest that ADA3 interaction may contribute to RARα transcriptional activity. Interestingly, most of these ADA3 interaction mutants also display dominant-negative effects as they suppressed the reporter gene expression below control level, possibly due to sequestration of endogenous cofactors. Although the contribution of other transcriptional co-activators, such as the p160 proteins, cannot be undermined, these results support the idea of ADA3 as a co-activator for RARα.
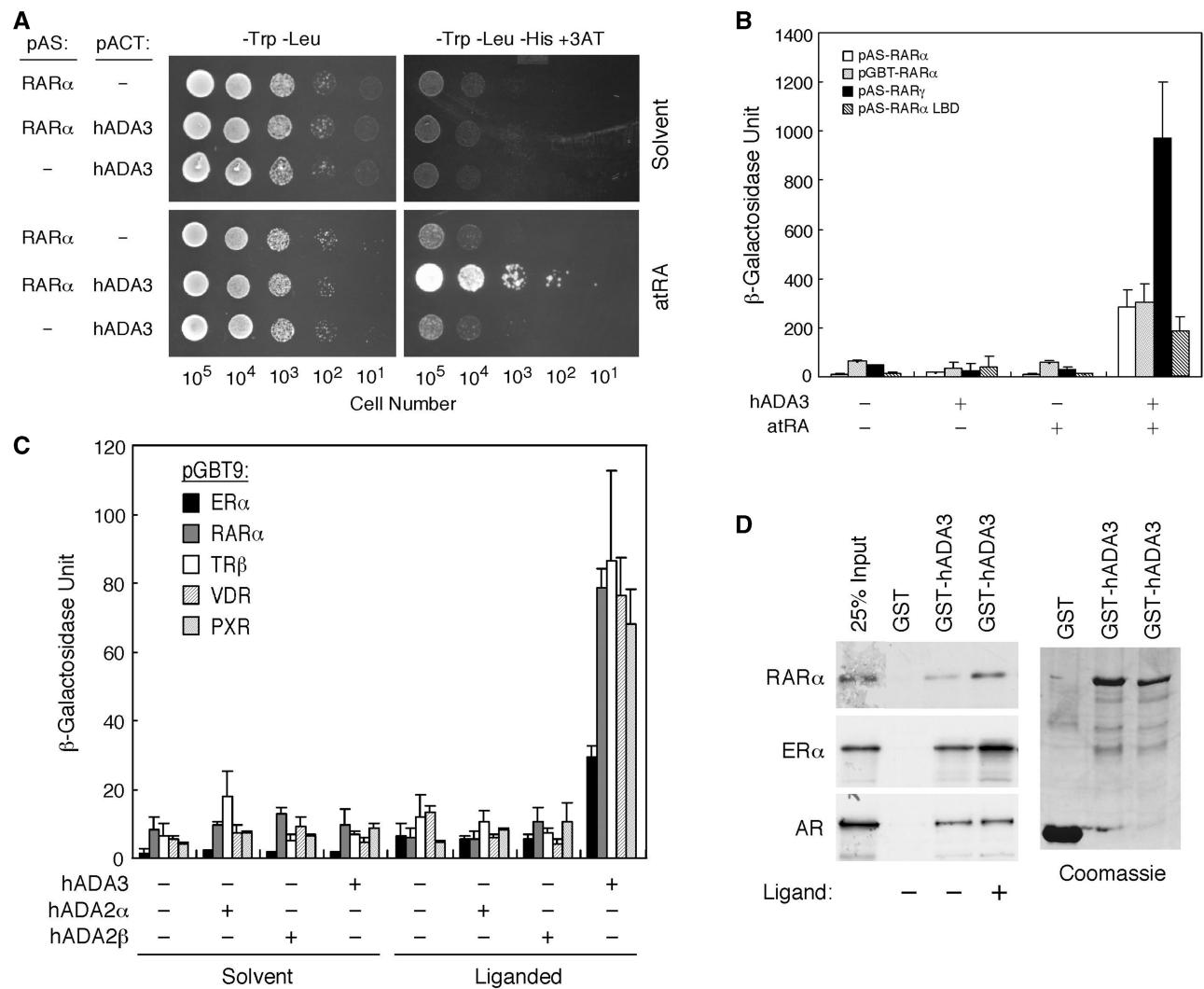
Figure 2. ADA3 binds to RARα in a ligand-dependent manner. (**A**) Yeast two-hybrid assay showing ligand-dependent RARα-ADA3 interaction. Full-length hRARα was expressed as GAL4 DBD fusion from pAS1 vector, while full-length hADA3 as GAL4 AD fusion from pACT2 vector. Yeast Y190 cells harboring the indicated plasmids were spotted onto SC-Trp-Leu or SC-Trp-Leu-His+3AT (20 mM) plates, in the absence (solvent) or presence of atRA (100 nM). The numbers of cells spotted are shown at the bottom. (**B**) Interaction of hADA3 with various RAR plasmids in yeast two-hybrid assay. Yeast transformants containing indicated combinations of the specified RAR plasmid and pACT-hADA3 were grown on SC-Trp-Leu media supplemented without (−) or with (+) 1 μM of atRA. (**C**) Ligand-dependent interactions of ADA3 with various NRs in yeast two-hybrid assay. Y190 cells were transformed with the GAL4 DBD fusion of various indicated NRs together with the indicated GAL4 AD fusion of hADA3, hADA2α or hADA2β. Transformants were grown in SC-Trp-Leu selection media in the presence or absence of cognate ligand (10 nM E2 for ERα, 10 nM atRA for RARα, 10 nM T3 for TRβ, 10 nM vitamin D3 for VDR and 10 μM rifampicin for PXR). (**D**) Interactions of hADA3 with NRs *in vitro* by GST pull-down assay. Purified GST proteins were incubated with [^{35}S]-labeled hRARα, hERα or hAR without or with ligands (1 μM atRA for hRARα, 100 nM E2 for hERα and 100 nM DHT for hAR).

Putative LxxLL motifs of ADA3 are involved in interaction with RARα

Several NR co-activators, including the p160 and DRIP220, utilize highly conserved LxxLL motifs to interact with the co-activator-binding pocket of the receptor. Previously, two putative LxxLL motifs were identified in the mouse ADA3, although they were excluded from mediating interaction with NRs (25). As shown in Figure 5A, we have located five putative LxxLL consensus sequences in the hADA3 sequence. We tested whether these putative LxxLL motifs are involved in interaction with RARα. Site-directed mutations of conserved hydrophobic amino acids within each

motif were created and tested for interaction with liganded RARα in a yeast two-hybrid assay. Intriguingly, alterations of NR box 3 (mNR3), 4 (mNR4) and 5 (mNR5) showed diminished interaction with RARα in the presence of atRA (Figure 5B). In contrast, alterations in NR boxes 1 (mNR1) and 2 (mNR2) had little effect. As a control (Figure 5C), none of these NR box mutations of ADA3 affected its interaction with ANCO-1 (32). These results suggest that hADA3 may utilize specific LxxLL motifs for interaction with the co-activator pocket of RARα. Additionally, the effect of NR box mutation on the hRARα co-activator function of ADA3 was investigated by transient transfection reporter gene assay. Consistently,
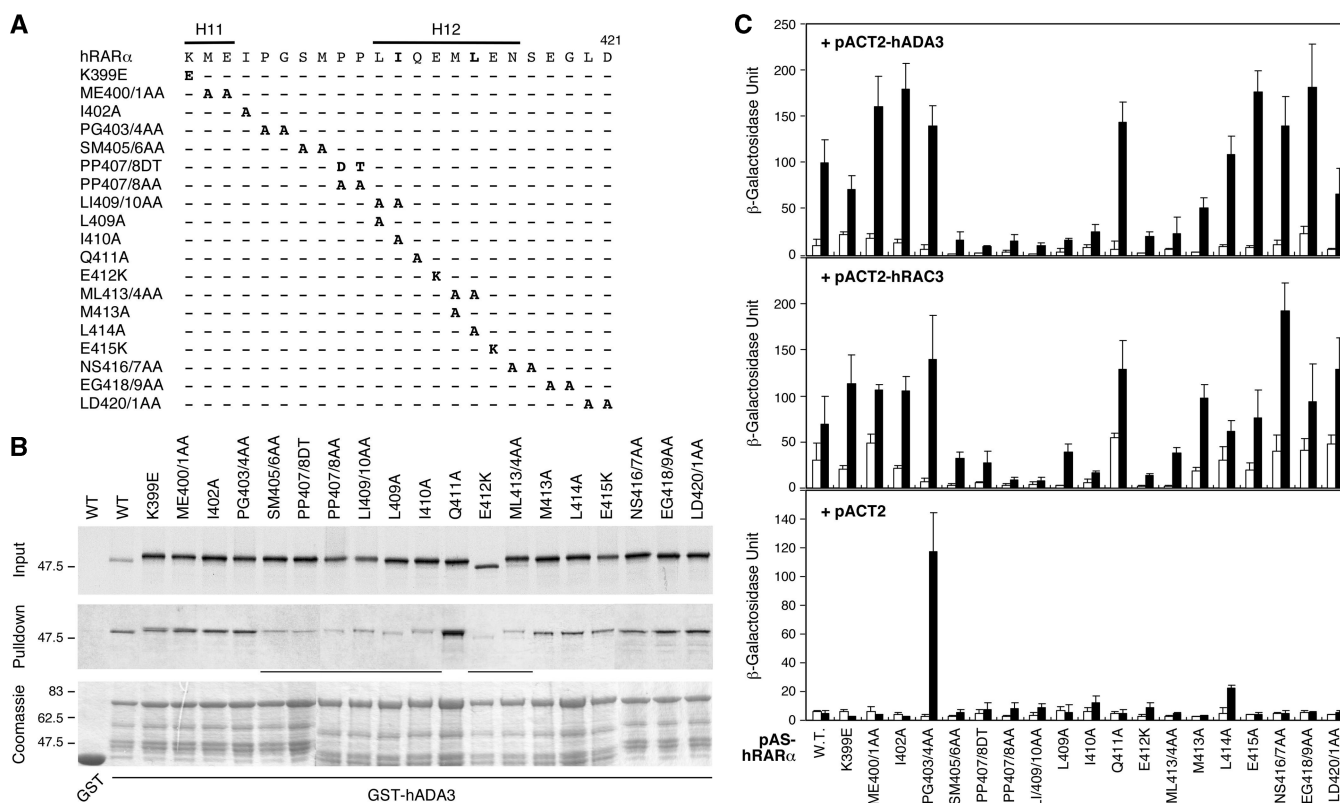
Figure 3. ADA3 interacts with the co-activator pocket of RARα. (**A**) Summary of site-directed mutants surrounding hRARα H12 helix. Substituted amino acids for individual mutations are as indicated at the mutated residues. (**B**) A GST pull-down assay showing interactions of GST-hADA3 with [³⁵S]-hRARα and its mutants. Approximately 20% of the labeled proteins used in the pull-down reaction are shown in the input panel. The pull-down panel shows the amount of labeled RARα that interacted with GST-hADA3 or GST alone (first lane) in the presence of 1 μM of atRA. (**C**) Comparison of the interactions of RARα H12 mutants between hADA3 and RAC3 in yeast two-hybrid assay. Yeast Y190 cells were co-transfected with each indicated pAS-hRARα construct shown at bottom together with pACT-hADA3, pACT-RAC3 or pACT2 empty vector. The transfected cells were grown in SC-Trp-Leu media containing either solvent (open bars) or 100 nM of atRA (solid bars) for 36 h. The β-galactosidase units reflect interactions between various hRARα constructs with empty vector, RAC3 or ADA3, respectively.
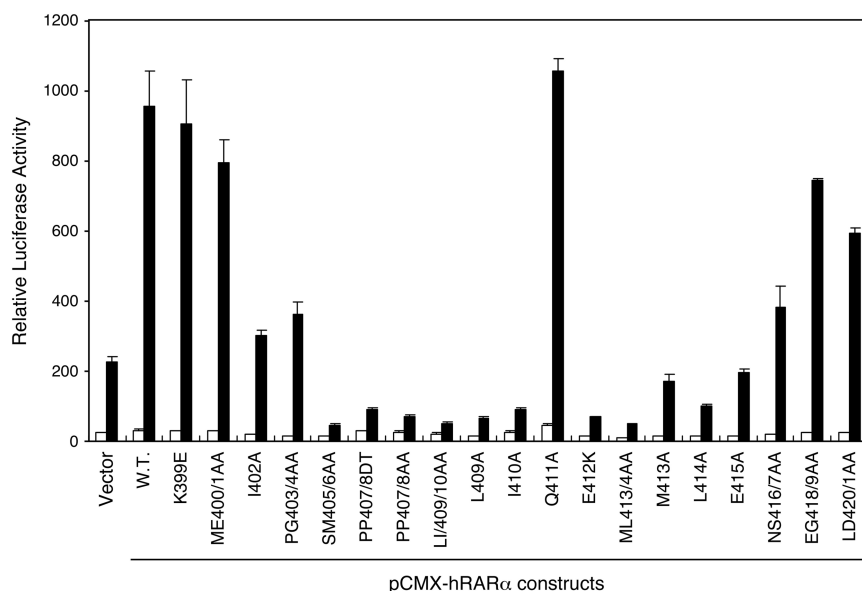

Figure 4. ADA3 interaction deficiency mutants of RARα display impaired transcriptional function in mammalian cells. The transcriptional activities of the hRARα H12 mutants shown in Figure 3A were determined by transient transfection reporter assay in HEK293 cells. Each of the indicated pCMX-hRARα constructs was cotransfected with the pDR5-tk-Luc reporter and the β-galactosidase control plasmid. Transfected cells were treated with either solvent (white bars) or 50 nM atRA (black bars). The relative luciferase activities were determined from three independent samples after normalization to the internal β-galactosidase control.
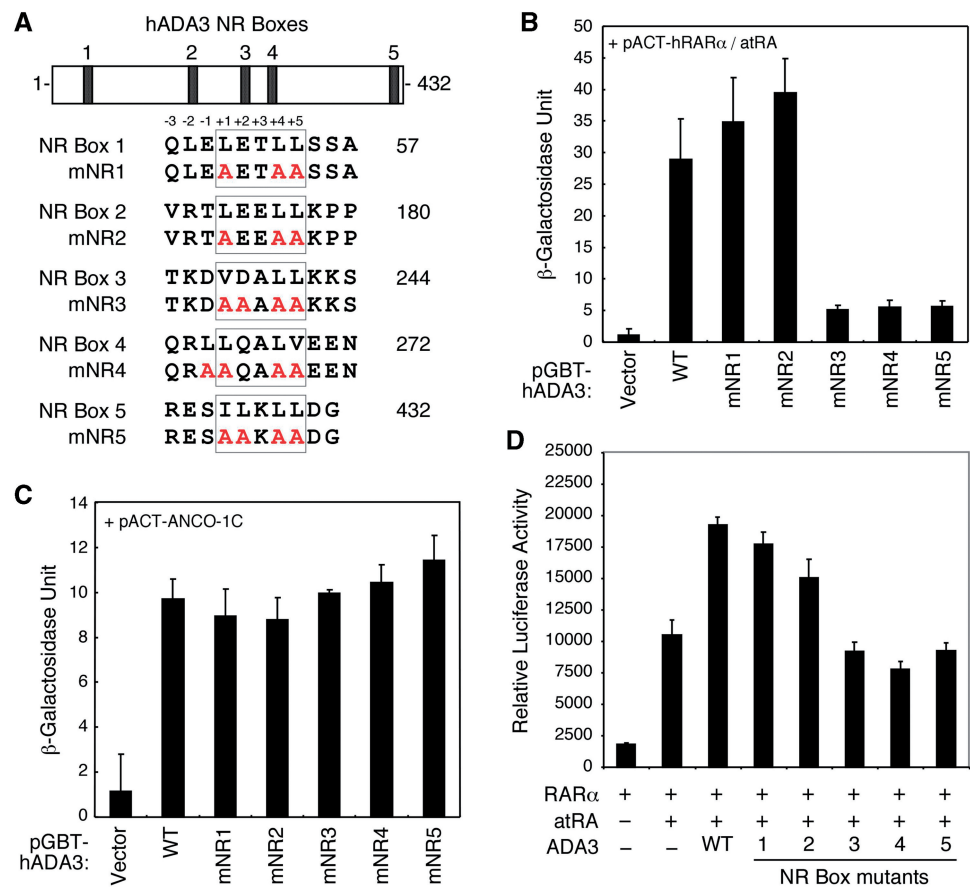
Figure 5. Three NR boxes in ADA3 are required for RARα interaction. (**A**) Amino acid alignment of the five putative hADA3 NR boxes and their corresponding mutants. Relative positions of individual NR boxes are marked in the hADA3 schematic structure on top. The amino acid positions relative to the LxxLL core motif are shown on top of the peptide sequence. (**B**) Yeast two-hybrid interactions of pACT-hRARα with pGBT-hADA3 wild-type or NR box mutants. Vector indicates blank pGBT9 vector. Cells were treated with 50 nM atRA for 24 h before β-galactosidase assay. Mutations of NR boxes 1 and 2 had no effect on RARα interaction, while mutations of NR boxes 3, 4 and 5 abolished the interaction. (**C**) Yeast two-hybrid interactions of pACT-ANCO-1C with pGBT-hADA3 wild-type and NR box mutants. None of the ADA3 NR box mutations affected the interaction between hADA3 with ANCO-1. (**D**) ADA3 NR boxes 3, 4 and 5 mutations abolished the coactivation function of hADA3 on hRARα. HEK293 cells were cotransfected with pCMX-hRARα along with indicated pCMX-hADA3 constructs. Transfected cells were treated with 100 nM atRA or solvent as control. Relative luciferase activity was determined from three independent experiments after normalization with co-transfected β-galactosidase internal control.

NR boxes 3, 4 and 5 mutants failed to enhance transactivation of hRARα (Figure 5D). These results suggest that the three NR boxes of hADA3 are involved in interaction and coactivation with hRARα.

'Gain-of-function' mutants and association of ADA3 with RAR target genes

The three putative NR boxes of ADA3 that are involved in interaction with RARα are atypical LxxLL motifs that may not be optimal for binding NRs. Therefore, we mutated the NR box 3 sequence from VDALL to LDALL (creating the V237L mutant), the box 4 LQALV to LAQLL (creating the V269L mutant) and the box 5 ILKLL to LLKLL (creating the I426L mutant). These potential 'gain-of-function' mutations were tested for interaction with hRARα in yeast two-hybrid assay. In this experiment, we found that all three mutations enhanced the interaction of ADA3 with liganded hRARα, although the effect of NR box 4 mutation was

less significant (Figure 6A). Together with the 'loss-of-function' mutants, these 'gain-of-function' mutants strongly indicate that ADA3 indeed interacts with hRARα via a mechanism involving NR boxes.

Furthermore, to determine if hADA3 could be recruited to RAR target genes, chromatin immunoprecipitation (ChIP) assay was performed. We transfected FLAG-tagged hRARα and HA-tagged hADA3 into both HEK293 and HeLa cells and conducted ChIP assays using monoclonal anti-FLAG and -HA antibodies on the nature hRARβ2 target promoter. In this experiment, the hRARβ2 promoter region that contains RAR responsive element was immunoprecipitated as expected with the anti-FLAG antibody, but not with the control IgG (Figure 6B). The same hRARβ2 promoter region was also immunoprecipitated with anti-HA antibody, but this occurred only in the presence of HA-hADA3 co-transfection and atRA treatment. Similar results were obtained in both HEK293 and HeLa cells. These data strongly suggest that ADA3 indeed can associate with
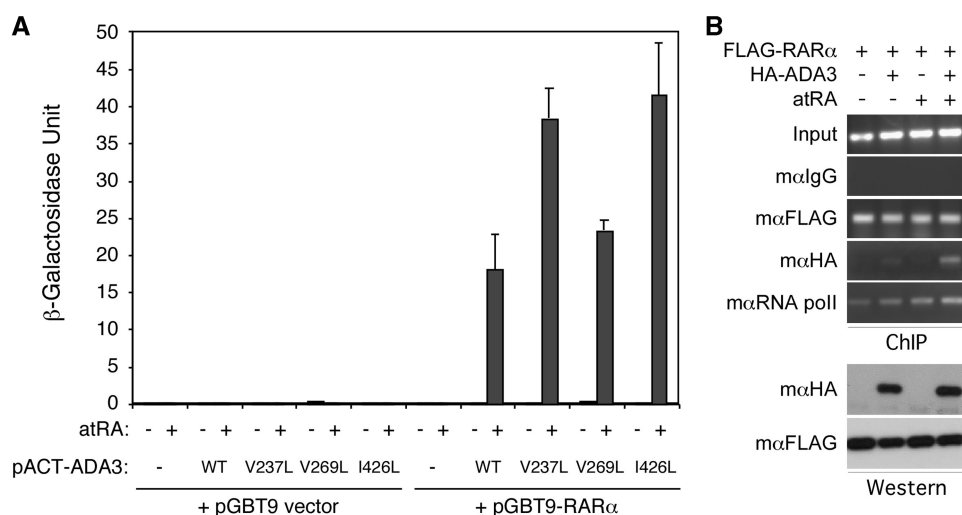
Figure 6. (**A**) 'Gain-of-function' mutations of ADA3 NR boxes enhance interaction with RARα. We mutated the ADA3 NR Box 3 VDALL to LDALL (V237L), Box 4 LQALV to LQALL (V269L) and Box 5 ILKLL to LLKLL (I426L). The mutations were generated on the pACT-ADA3 construct and tested for interaction with pGBT9-RARα in yeast two-hybrid assay. The pGBT9, pACT2 (−) vectors and the pACT-ADA3 wild-type (WT) were used as controls. The transformed colonies were grown in SC-Trp-Leu media containing either solvent (open bars) or 100 nM of atRA (solid bars) for 36 h and β-galactosidase activities were determined from three independent cultures. (**B**) ADA3 associates with RARβ2 promoter in a hormone-dependent manner. Transiently transfected HEK293 cells were serum-starved and then stimulated with or without 2 μM atRA for 3 h. The cells were subjected to *in vivo* ChIP assay. The FLAG, HA and RNA polymerase II antibodies were used in the immunoprecipitation. Mouse IgG was used as a negative control, whereas input chromatin was used as loading control for the PCR reactions.

RAR target gene promoter *in vivo* in a hormone-dependent manner.

A structural model of the ADA3/RARα complex

To corroborate on the findings that the LxxLL motifs of ADA3 and the co-activator pocket of RARα are involved in their interaction, we employed a molecular modeling technique to deduce the most favorable structural model of the hADA3 NR box 4 peptide complexed with the hRARα LBD. An existing crystal structure of the hRARβ/TRAP220 complex (31) was used as a molecular template for building the hRARα/ADA3 NR box 4 peptide structural model. The ADA3 NR box 4 (QRLLQALVEE) was chosen because it is essential for interaction with RARα and bears the highest similarity (70%) to the TRAP220 NR box 2 (RHKILHRLLQEGS) used in the crystal study (31). The resultant model was further refined using MD and energy minimization. The sequence of the NR box 4 was modeled as a short two-turn α-helix. The average structure derived from a 2 ns MD simulation indicates that the ADA3 peptide mimicked the observed binding mode of the LxxLL motif to other NR LBDs, and interacted with the RARα LBD through a hydrophobic groove formed by helices 3, 4, 5 and 12 (Figure 7A). The proper position of the peptide was locked by two conserved charge clamp residues, Lys244 and Glu412 positioned at opposite ends of the cleft via their electrostatic interactions with backbone atoms on the ends of the motif helix. A complex network of hydrogen bonds is formed between the amino-terminal residues of the peptide and the surrounding residues on the receptor (Figure 6B). In addition to the classical charge clamp interaction between Glu412 and the amide nitrogen atom of Leu-1, two additional electrostatic

interactions are formed: one between Lys262 and Gln-3 and another between Asn416 and Arg-2. These two electrostatic interactions may explain the observed differential influence of mutated NR boxes and their interactions with hRARα (Figure 5). This hydrogen bond network can only form in NR boxes 3, 4 and 5, which augments the van der Waals contacts at the core hydrophobic interface and helps stabilize the protein–peptide complex. However, these interactions are missing from NR boxes 1 and 2 due to the presence of hydrophobic residues at positions -2 and -3, respectively. Compared with the binding of the LxxLL motif from TRAP220 to hRARβ, the helix formed by ADA3 NR box 4 is shifted toward H12 by ~1.7Å (Figure 6C and D). Side chains of several residues undergo conformational changes upon binding of the ADA3 peptide. The carboxyl group of Glu412 is rotated ~180 degrees around the C_B–C_A bond in order to accommodate the shift of the ADA3 peptide toward H12. In this binding mode, residues of Leu+1, Leu+4 and Val+5 of the ADA3 peptide comprise the core hydrophobic interface with the corresponding hydrophobic groove of the RARα co-activator pocket. Their predicted interacting residues on the receptor are listed in Table 1.

DISCUSSION

In this study, we have determined the role of human ADA3 in RARα-mediated transcriptional activation and investigated the underlying molecular mechanisms. We show that hADA3 interacts directly with hRARα and enhances its transactivation function. The interaction between ADA3 and RARα is ligand-dependent. RARα interacts specifically with ADA3 but not other ADA subunits, while ADA3 may interact with other NRs.
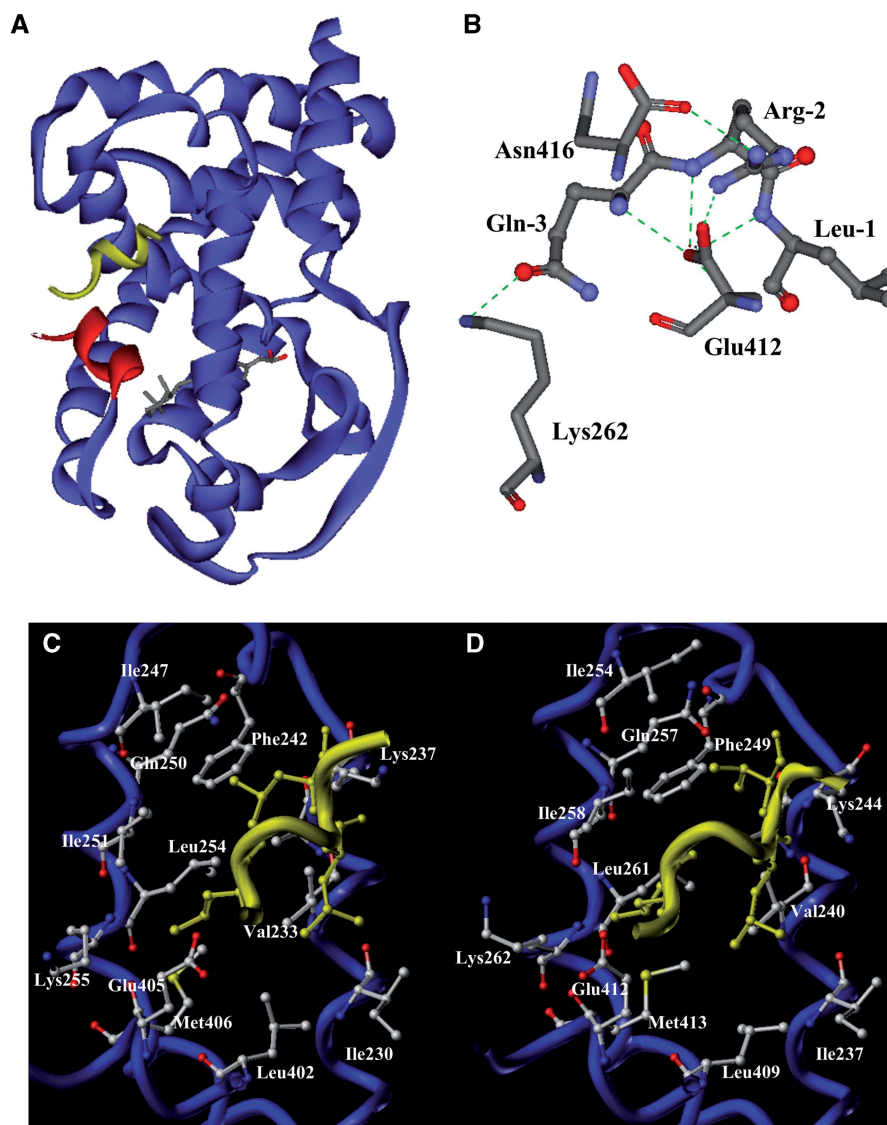
Figure 7. A structural model of the ADA3 NR box 4/RARα LBD complex. (**A**) An overview of the RARα-ADA3 NR box 4 complex. The RARα LBD is shown as blue ribbons except helix 12 is colored in red. The peptide from ADA3 NR box 4 is shown in yellow. (**B**) Hydrogen-bond interaction map around the N-terminal of the ADA3 NR box 4 peptide. The green dashed lines represent hydrogen bonds. The three N-terminal residues from ADA3 peptide Leu-1, Arg-2 and Gln-3, along with the Lys262, Glu412 and Asn416 residues from RARα, are shown as indicated. (**C, D**) Structural comparison between hRARβ/TRAP220 and hRARα/ADA3 complexes. Helices 3, 4, 5 and 12 are displayed as blue coils and co-activator motifs are colored in yellow. Residues on the receptor interacting with peptides are depicted in stick representation and colored by atom-type (carbon: grey; nitrogen: blue; oxygen: red). The residues from peptides are colored in yellow and, for clarity, only residues making significant interactions with the LBD are shown. (**C**) Structure of the RARβ/TRAP220 NR box 2 complex. (**D**) Structure of the RARα/ADA3 NR box 4 complex.

Table 1. The hRARα residues predicted to make contacts with the core hydrophobic residues of hADA3 NR box 4

Helix	Residues
Helix 3	Ile237, Val240, Ala 243, Lys244
Helix 4	Phe249
Helix 5	Ile254, Gln257, Ile258, Leu261, Lys262
Helix 12	Leu409, Glu412, Met413

The hRARα residues are listed according to their relative locations within indicated helices of hRARα LBD

Several crucial amino acid residues on the transactivation helix (H12) of RARα co-activator pocket are identified as essential for the interaction with ADA3, as well as for the transactivation function of RARα. Furthermore, three putative LxxLL NR boxes of ADA3 are found to be critical for interaction with liganded RARα, and ADA3 is capable of associating with RAR target gene promoter in a hormone-dependent manner. We propose a structural model of the ADA3 NR box 4/RARα LBD complex to illustrate the mode of interactions. These data suggest that hADA3 is involved in RARα-mediated transactivation,

likely through a mechanism of direct physical interactions between the NR boxes of ADA3 and the co-activator pocket of liganded RARα.

The involvement of ADA3 in NR signaling was first demonstrated in *Saccharomyces cerevisiae* when yeast ADA3 was isolated as an RXRα-interacting protein (24). The yeast ADA adaptor complex is also important for transcriptional activation by GR (33,34) and TR (35), where the yeast ADA2 seems to play a more important role. The yeast ADA3 is thought to mediate transactivation by RXRα and ERα in yeast cells through direct physical interaction and recruitment of the SAGA complex (24). In that study, yeast ADA3 was shown to interact in a ligand-dependent manner with RXRα, TRα and ERα, but not with RARα. Surprisingly, the same group reported that mouse ADA3 does not interact with NRs, even though it contains potential LxxLL motifs (25). Inconsistent data were reported regarding the roles of human and mouse ADA3 in terms of their interactions and function with human ERα/β and RXRα and hRARα in mammalian cells (22,26). Interestingly, yeast ADA3 was implicated in mediating RARα transactivation through interaction with the p160 co-activator RAC3/AIB1 and recruitment of GCN5 co-activator complex (27). Our current results demonstrate a strong ligand-dependent direct interaction between hADA3 and RARα *in vivo* and *in vitro*. We show that human ADA3, but not ADA2α or β, is capable of interaction with several NRs including ERα, TRβ, VDR, PXR and AR. This is consistent in part with prior reports supporting a direct role of hADA3 in ER signaling (22,26). However, it is noted that ADA2, instead of ADA3, was previously implicated in TRβ signaling (35), although our current data suggest that TRβ interacts with hADA3 but not ADA2α or 2β (Figure 2C). To our surprise, ADA3 seems to bind to RAR subtypes with different affinity. In particular, RARγ shows a 3-fold better interaction with ADA3 as compared to RARα (Figure 2B). We reason that the ω loop in the RARγ, which is absent in RARα and RARβ, might facilitate the conformational change of the RAR/ADA3 interaction. Since RARα and RARβ have been shown to be associated with the development of acute promyelocytic leukemia and squamous cell cancers, whereas RARγ is associated with retinoid effects on mucocutaneous tissues and bone, our results suggest that ADA3 may be involved in multiple physiological effects mediated by different subtypes of RARs. While this study focuses on dissecting the molecular interaction between ADA3 and RARα, the attribution of RAR target gene expression to different co-activators has been an important, yet challenging topic, especially due to the existence of overlapping co-activators, and the fact that cell is able to compensate the loss of a particular co-activator. This is exemplified in our ADA3 knockdown experiment where RAR reporter gene expression was affected but not abolished (Figure 1B).

To corroborate on the role of human ADA3 in RARα signaling, we have investigated the molecular basis of their interactions. Several co-activators, including the p160 proteins, are known to utilize a simple LxxLL NR box for interaction with a structurally conserved co-activator pocket located on the surface of a receptor LBD (12,29,36,37). The co-activator pocket is formed by helices 3, 4, 5 and 12 in response to ligand-induced conformational change, with helix 12 serving as a dynamic lip that closes up upon ligand binding (28,38). Our data demonstrate that the co-activator pocket of RARα is indeed required for ADA3 binding. In addition, we have identified three potential LxxLL motifs in hADA3, whose disruption clearly abolished the interaction between ADA3 and RARα (Figure 5). Importantly, 'gain-of-function' mutations of these NR boxes that optimize the NR-binding potentials of the LxxLL motif enhanced interaction with RARα (Figure 6A). The fact that the optimization of NR box 4 consensus sequence has the least effect on enhancing interaction with RARα is consistent with the idea that this motif is nearly optimal in interaction with RARα as demonstrated by molecular modeling (Figure 7). Whether these NR boxes are essential for interactions with other NRs, and the relative importance and the stoichiometry of these motifs, remain to be determined. The reason that a single NR box mutation in ADA3 abrogated interaction with RAR may be multifaceted. One plausible explanation is that all three NR boxes are involved in the interaction, while each of them is required for the formation of a stable complex *in vivo*. Thus, mutation in a single NR box would result in the destabilization of the ADA3/RAR complex.

Given that ADA3 is a key subunit of PCAF co-activator complexes, it is reasonable to speculate that ADA3 may act as a molecular bridge to mediate the recruitment of co-activator complexes to ligand-activated receptors. Our data show that not only the transcriptional activity of RARα was enhanced by overexpression of ADA3, silencing of endogenous ADA3 also diminished reporter gene expression (Figure 1). Therefore, we suggest that ADA3 may indeed function as a transcriptional co-activator for RARα. We further speculate that ADA3 may play a role in bringing its associated-co-activator complexes to other NRs. Nonetheless, there are likely receptor and receptor-subtype selectivity for ADA3, because its interaction with AR was independent of ligand and a better interaction with RARγ subtype was observed. Despite the positive epigenetic regulation of gene transcription, ADA3 also directly binds and acetylates P53, reducing Mdm2-mediated ubiquitination of P53 and subsequently stabilizing p53 expression (39). In this regard, ADA3 may also function as an RAR acetyltransferase and affect its protein turnover rate. Various possibilities warrant detailed study and will be addressed in future work.

The co-activator binding characteristics of RARα between ADA3 and RAC3 were analyzed and compared using a series of systematic helix 12 mutations in RARα (Figure 3). Intriguingly, the interacting pattern is highly related to RARα transactivation capability (Figure 4), suggesting that RARα activity is controlled by these cofactors. Additionally, the lack of ADA3 or RAC3 binding seems to correlate with a dominant negative function of RARα, possibly due to sequestration of endogenous cofactors such as RXR. This observation is consistent with a prior finding where disrupting TRβ and SRC1 interaction

resulted in a dominant negative regulation in TRβ (40). Similarly, the transcriptional regulation of Hepatocyte Nuclear Factor-4α is highly dependent on its physical interaction with PGC-1 and SRC3 (41). Thus, we hypothesize that ADA3 utilizes a similar binding pocket as p160 co-activators in regulating RAR signaling. While ADA3 and p160 co-activators share the same binding property to RARα, it is conceivable that these two co-activators may compete with each other if present at the same time. It will be of special interest to know whether there is specific physiological condition(s) that governs a potential binding switch from p160 to ADA3, or vice versa.

With the help of molecular modeling, we have created a structural model of the hADA3 NR box 4 peptide/hRARα LBD complex (Figure 7). This thermodynamically stable complex is reminiscent of the TRAP220 NR box 2/hRARβ LBD complex that was previously solved by X-ray crystallography (31). Similar to other co-activator complex, the primary interaction comes from the three core hydrophobic residues of the LxxLL motif, whose side chains interpolate deeply into the co-activator pocket. Many hydrophobic contacts are formed with residues from RARα LBD helices 3, 4, 5 and 12 (Table 1). Nearly all of these residues are conserved amongst different NRs and required for binding with other co-activators (28,29,36,38,42,43). In agreement with this model, our data show that the three predicted contacting residues in H12, Leu409, Glu412 and Met413 are all required for ADA3 interaction (Figure 3). Similarly, Val240 in helix 3, Phe249 in helix 4 and Leu261 in helix 5 are also important for ADA3 binding (data not shown). It is interesting to note that additional residues within and around H12 are also important for ADA3 binding. Based on this model, a complex hydrogen bond network is predicted between the N-terminal of the NR box 4 peptide and the surrounding residues on the receptor (Figure 7B). Importantly, two additional electrostatic interactions between Arg-2 and Asn416 and Gln-3 and Lys262 may explain the differential affinity of the five LxxLL sequences for their interaction with RARα. The Leu-2 and Val-3 residues on NR box 1 and 2, respectively, may prevent formation of hydrogen bond, thus this hydrogen bond network may only occur with NR boxes 3, 4 and 5.

Taken together, we have provided strong evidence that support an important role of hADA3 as a transcriptional co-activator for RARα in mammalian cells. These data suggest a mechanism through which the mammalian ADA3 may direct the action of multi-functional co-activator complexes toward modulating NR signaling.

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